SPATIAL REGULATION OF THE POLARITY PROTEIN APKC DURING ASYMMETRIC CELL DIVISION OF *DROSOPHILA* NEUROBLASTS

by

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DISSERTATION ABSTRACT

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The Par complex protein, atypical protein kinase C (aPKC), plays an instrumental role in diverse cell polarities. aPKC is able to restrict substrate localization through a phosphorylation-induced cortical exclusion mechanism, allowing for the generation of molecularly distinct cortical domains. Thus, controlling the localization of aPKC is central to Par-mediated polarity but the mechanism by which aPKC is polarized remains poorly understood. In this dissertation I investigated the restriction of aPKC to the apical cortex of *Drosophila* neural stem cells, neuroblasts, as these cells dynamically polarize aPKC through repeated asymmetric cell divisions. The polarity created through aPKC phosphorylation must be tightly regulated in order to ensure proper balance between self-renewal and differentiation.

To begin, I investigated whether or not aPKC's so called 'maturation' by PDK1 phosphorylation is required for aPKC activity and localization. We found that aPKC's phosphorylation by PDK1 is required for both polarity and full activity. An aPKC containing an unphosphorylatable activation loop mutation localizes symmetrically around the cortex in a manner independent of its binding partner, Par-6, suggesting that aPKC could interact with the cortex by an unknown mechanism.

To investigate how aPKC is able to localize to the cortex independent of Par-6, I

used an *in vivo* structure function analysis of domains within aPKC, accompanied by biochemical approaches. I identified a necessity for the aPKC C1 domain for binding to the neuroblast cortex. This interaction is mediated by negatively charged phospholipids. Neither aPKC interaction, with phospholipids or Par-6, is sufficient to restrict aPKC to the apical cortex. Thus, aPKC polarization utilizes a dual interaction mechanism that takes advantage of both protein-lipid and protein-protein interactions, and proper control of each of these signals is required to prevent neuroblast division defects. One interaction, mediated by the C1, is a general cortical targeting mechanism, whereas the other specifies polarization mediated by Par complex interactions. We conclude that a conformational change induced by these interactions activates aPKC's catalytic activity, thereby coupling localization and activity.

This dissertation includes unpublished co-authored material.

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MOLECULAR CONTROL OF ATYPICAL PROTEIN KINASE C: TIPPING THE BALANCE BETWEEN SELF-RENEWAL AND DIFFERENTIATION

Chapters I, II, and III contain unpublished co-authored material.

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INTRODUCTION

Complex organisms are faced with the challenge of generating and maintaining diverse cell types, ranging from simple epithelia, to neurons and motile immune cells (Clevers et al., 2014; Kohwi and Doe, 2013; Seita and Weissman, 2010). To meet this challenge, a complex set of regulatory pathways controls nearly every aspect of cell growth and function, including genetic and epigenetic programming, cytoskeleton dynamics, and protein trafficking. Given the far reach of cell fate specification pathways, it is not surprising that their malfunction can be catastrophic for both development and tissue homeostasis in adult organisms. Furthermore, the promise of stem cells as a therapeutic derives from their ability to deftly navigate the multitude of pathways that control cell fate (Chen et al., 2013, 2013; Kreso and Dick, 2014; Segers and Lee, 2008). Our understanding of the molecular components that make up these pathways has increased dramatically, and recent work has uncovered a key role for the atypical Protein Kinase C (aPKC), best known as a member of the Par polarity complex, as a key regulator of cell fate decisions in metazoans (Doe, 2008; Homem and Knoblich, 2012;

Tepass, 2012). Here we review what is known about how aPKC is controlled, how aPKC controls downstream factors to specify cell fate, and the functional interplay between polarity and cell fate specification.

PKC family kinases are ubiquitous components of cellular signaling pathways. Although they have long been thought to be oncogenes, the recent discovery that inactive PKCs are correlated with cancer indicates that they may instead be tumor suppressors (Antal et al., 2015). In animals, PKCs are commonly divided into three subfamilies (yeast contain a single PKC), including the conventional, novel, and atypical (Newton, 2010). This last group contains the iota and zeta isoforms in mammals, and a single isoform in flies and worms. All family members contain a catalytic domain at the COOH-terminus connected to NH_2-terminal regulatory domains. Each isoform is specialized by their kinase domain's specificity, which determines the repertoire of substrates that they can phosphorylate, and the regulatory elements that determine when and where substrates will be phosphorylated(Newton, 2010) (Figure 1.1A). For example, diacylglycerol (DAG) activates the conventional and novel isoforms, leading to phosphorylation of a distinct set of substrates, but DAG does not activate atypical PKCs (Colón-González and Kazanietz, 2006; Giorgione et al., 2006; Newton, 2010; Pu et al., 2006). The somewhat unique role of aPKC in cell fate specification arises from these specializations: the kinase domain phosphorylates targets important for cell fate, and the regulatory elements are responsive to upstream components that coordinate cell fate changes (Doe, 2008; Homem and Knoblich, 2012; Prehoda, 2009). Here we focus on recently discovered aPKC substrates that regulate cell fate, and progress in understanding how aPKC activity is coordinated with cell fate decisions.

Although aPKCs have a different complement of upstream regulators compared to their conventional and novel counterparts, they share several important regulatory elements (Figure 1.1A). Perhaps most important is the "pseudosubstrate", which has many of the sequence characteristics of a normal substrate so that it can bind in the kinase domain active site, but an alanine at the position that would be phosphorylated prevents progression through the catalytic cycle (Graybill et al., 2012; Newton, 2010). Determining how the pseudosubstrate is removed from the kinase domain's active site is a key part of understanding PKC activation mechanisms, but other domains, such as the C1, may directly repress kinase activity (Graybill et al., 2012; Lopez-Garcia et al., 2011). The C1 cysteine rich domain is directly COOH-terminal to the pseudosubstrate in all PKCs, and in the single structure of a full-length PKC, the C1 binds a lobe of the kinase domain where it can inhibit activity (Leonard et al., 2011). aPKCs is distinguished from the other family members by the presence of a PB1 domain that heterodimerizes with certain PB1s from other proteins, and a COOH-terminal PDZ ligand sequence (Hirano et al., 2004; Lamark et al., 2003; Newton, 2010; Noda et al., 2003). The mechanisms by which these domains are used to control aPKC's activity are just now being uncovered.

PKCs are also commonly regulated by post-translational modification (Figure 1.1A). A series of two or three phosphorylations within their kinase domain is required for activity (Borner et al., 1989). These phosphorylations occur at three distinct places: the activation loop, the turn motif, and the hydrophobic motif (Newton, 2010) (Figure 1.1A). Phosphoinositide-dependent kinase 1 (PDK1) phosphorylates the activation loop, whereas the turn motif is autophosphorylated (Chou et al., 1998; Dutil et al., 1998; Newton, 2010). aPKC isoforms possess a phosphomimetic residue at the hydrophobic

motif, and therefore it does not require phosphorylation of this residue for activation. The role of these phosphorylations in regulating aPKC is unclear, as structural evidence suggests that they may not be required for activity (Wang et al., 2012).

As a regulator of cell polarity, perhaps its most-studied function, aPKC is a member of the Par (partitioning defective) complex, which includes Par-3 (Bazooka in flies), and Par-6 (Goldstein and Macara, 2007; Suzuki and Ohno, 2006). Polarity is essential for many aspects of cell function, and as described below, can also be critical for cell fate specification. The Par complex is found in diverse polarized cell types to create mutually exclusive cortical domains (Prehoda, 2009; Tepass, 2012) (Figure 1.1B). In the current model for Par complex function, aPKC is a key output of Par complex activity as it phosphorylates downstream targets to displace them into the cytoplasm (Prehoda, 2009). These substrates can localize to cortical regions that lack the Par complex but are removed from the cortex once they enter the Par domain (Figure 1.1B). Thus, two key aspects of polarity are ensuring active aPKC is targeted to the proper cortical areas and coupling substrate phosphorylation to cortical displacement. Although these are fundamental questions in polarity, our understanding of both is still in its infancy. Furthermore, as described below, aPKC's role in fate specification appears to involve aspects of its function that are independent of polarity regulation.

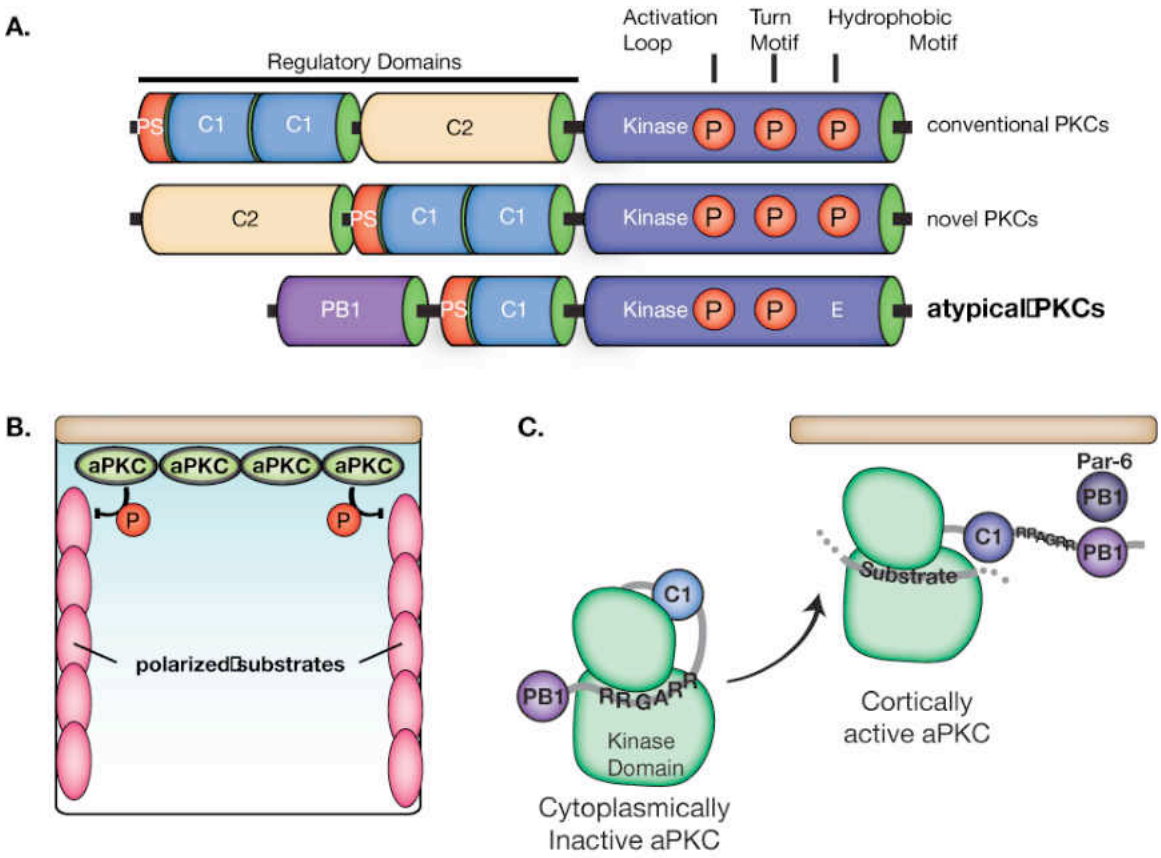


Figure 1.1. PKC family kinases, regulation and function of atypical Protein Kinase C. (A) Schematic of the protein kinase C family showing domain architectures, demonstrating both common and unique aspects of each PKC family member (PS = pseudosubstrate; C1 and C2 are cysteine rich domains; PB1 Phox/Bem1 domain). (B) Schematic of Par-mediated polarity mechanism. aPKC generates cellular polarity through phosphorylation and exclusion of cortically localized substrates (pink). (C) Activation of aPKC by Par-6 binding. Par-6's interaction with aPKC's PB1 domain disrupts the pseudosubstrate's inhibition of the kinase domain. The C1 domain may also play a role in regulating aPKC kinase activity.

Fate determinant segregation by aPKC during asymmetric cell division

Asymmetric cell division (ACD) is a highly dynamic process that generates daughter cells with distinct fates, typically with one daughter retaining the mother's fate and the other differentiating (Doe, 2008; Homem and Knoblich, 2012). It is aPKC's role in polarity that underlies the differential fates assumed by each daughter cell. This function has been most extensively studied in the *Drosophila* neuroblast (NB), a neural progenitor cell that is found in both the embryonic and larval nervous systems. Early in

mitosis, the NB begins to polarize such that by metaphase aPKC and the rest of the Par complex localize to one half of the cell cortex, while neuronal fate specification factors localize to the other half. Because the mitotic spindle is aligned with cortical polarity, the cytokinetic furrow bisects the two cortical domains: one daughter cell is formed from the cortex containing the Par complex, and the other forms from the cortex with differentiation factors bound.

NBs with incorrect levels of aPKC activity fail to asymmetrically divide and can exhibit characteristics of tumor cells (Homem and Knoblich, 2012). Excess aPKC activity leads to indefinite replication capacity (Caussinus and Gonzalez, 2005), whereas NBs with inadequate activity are unable to faithfully segregate cell fate determinates, leading to premature quiescence and differentiation (Lee et al., 2006a; Rolls et al., 2003). NB self-renewal and neuronal differentiation are both dependent on when and where aPKC is active, making the mechanisms that control aPKC localization and activity fundamental to asymmetric cell division.

The Rho GTPase Cdc42 is a key regulator of the Par complex in NBs and other polarized cells. GTP-bound Cdc42 interacts with the semi-crib and PDZ domain of Par-6 causing a conformational change, which is essential for aPKC polarization (Atwood et al., 2007; Garrard et al., 2003; Hutterer et al., 2004). Par-6 contains a PB1 domain that binds aPKC's PB1 and this interaction, via an unknown mechanism, displaces the pseudosubstrate from the kinase domain active site (Graybill et al., 2012; Noda et al., 2003) (Figure 1.1C). Par-6 is required to recruit aPKC to the cortex, where lipid binding can play a direct role in the activation of aPKC downstream of phosphatidylionositol 3-kinase (PI3K) by binding phosphatidylinositol 3,4,5-phosphate (PIP_3) (Bandyopadhyay

et al., 1999; Standaert et al., 1997, 2001). The lipid ceramide also activates aPKC by directly interacting with the kinase domain, an interaction that is important for junction formation in epithelia and signaling during cellular stress conditions (Bourbon et al., 2000; Wang et al., 2009). Coupling of aPKC protein-protein and protein-lipid interactions to activation provides an elegant mechanism for ensuring that aPKC is active at the right place and time. Cdc42 may also play a direct role in controlling aPKC's kinase activity as the Par-6 semi-CRIB and PDZ are important for full activation of aPKC by Par-6, further coupling aPKC localization and activity to the NB apical cortex (Atwood et al., 2007; Hutterer et al., 2004; Lin et al., 2000). Loss of either Par-3 or Par-6 leads to improper aPKC localization, defective asymmetric cell division, and improper development (Atwood et al., 2007; Petronczki and Knoblich, 2001; Rolls et al., 2003).

While Cdc42 and Par-6 are critical for increasing the amount of cortically localized, active aPKC, the neoplastic tumor suppressor Lgl is an important repressor of localization and activity that helps ensure the basal cortical domain remains free of aPKC (and therefore bound to neuronal fate determinants) (Betschinger et al., 2003; Lee et al., 2006b). The mechanism by which Lgl inhibits aPKC has remained enigmatic. In NBs lacking Lgl activity, aPKC activity is no longer restricted to the apical cortex leading to an increase in proliferation and a loss of apico-basal polarity(Lee et al., 2006b). aPKC counteracts Lgl's repression by phosphorylating it and displacing it into the cytoplasm (Betschinger et al., 2003). How Lgl inhibits aPKC's localization to the basal cortex remains unknown.

While aPKC interactions with the Par complex and Lgl may form the core elements of the asymmetric cell division regulatory machinery, other players have been

recently identified. Dynamin associated protein-160 (Dap160) regulates both aPKC localization and kinase activity (Chabu and Doe, 2008). It co-localizes with the Par complex at the apical cortex of dividing NBs and interacts with both aPKC and Par6. Dap160, through an unknown mechanism, also helps ensure that aPKC is properly polarized and does not enter the basal cortical domain. Other factors that control aPKC activity and localization include Clueless (Goh et al., 2013) and Canoe/afadin (Choi et al., 2013; Speicher et al., 2008), although the mechanisms are poorly understood.

Besides excluding neuronal fate determinants from the self-renewed NB, aPKC also plays a direct role in maintaining NB fate. The transcription factor Zif represses NB formation and in NBs lacking Zif aPKC is unpolarized (Chang et al., 2010). The *aPKC* gene contains Zif binding sites and Zif appears to repress *aPKC* expression. Furthermore, Zif is an aPKC substrate and phosphorylation prevents its entrance into the nucleus, forming a feedback loop that regulates aPKC expression and localization.

Activating aPKC at the NB apical cortex is critical for restricting neuronal fate determinants to the basal cortex. These proteins include the coiled-coiled protein Miranda with its cargo protein, the transcription factor, Prospero (Pros; Prox1 in mammals), and the translational regulator Brain Tumor (Brat; TRIM3 in mammals), as well as the Notch signaling regulator Numb (Doe, 2008; Homem and Knoblich, 2012). Following mitosis, these determinants induce conversion into a ganglion mother cell (GMC) by preventing self-renewal and promoting differentiation. Pros is a homedomain transcription factor that translocates to the GMC nucleus and activates genes that specify differentiation while repressing genes that are necessary for self-renewal (Doe, 2008). High Pros expression in NBs is sufficient to drive their differentiation (Bayraktar et al., 2010) while

intermediate levels induce quiescence (Lai and Doe, 2014). Differentiation is aided by the translational repressor Brat, which regulates important proliferation signals including Cyclin E, β-Catenin, dMyc, and Mad (Betschinger et al., 2006; Harris et al., 2011; Komori et al., 2014), and the repressor of Notch signaling, Numb (Knoblich et al., 1995; Lee et al., 2006a).

The role of aPKC in specifying fate determination during asymmetric cell division is primarily related to its function in regulating cell polarity. More recently, aPKC signaling has been found to directly control cell fate decisions through phosphorylation of cell cycle inhibitors, transcription factors, and growth pathway signaling. In this section we review these new findings and discuss how aPKC interfaces with important developmental pathways, Hedgehog, Wnt, and Jak/STAT to control stem cell fate decisions.

Regulation of the cell cycle by aPKC

Cell fate specification is usually tightly coupled to the cell division cycle. For example, in certain contexts a prolonged G1 cell cycle phase leads to differentiation, while a shortened G1 promotes proliferation (i.e. self-renewal) (Calegari and Huttner, 2003; Lange and Calegari, 2010). Recent evidence from the *Xenopus* neuroectoderm suggests that G1 is controlled in part by the inhibition of G1 specific cyclin/cdks (Lange et al., 2009). Although many aPKC functions involve its cytoplasmic activity, aPKC is found in the nucleus of progenitor cells in this tissue (Sabherwal et al., 2009) consistent with a role in transcriptional regulation. This seems to be the case for at least one cell-cycle regulating protein in *Xenopus* progenitor cells, p27xic. p27xic is a CIP/KIP protein

family of cyclin-dependent kinase inhibitors (CDKIs) that prevents the G1 to S transition by inhibiting cyclin-dependent kinase 2 (Cdk2) through binding and sequestering it from the nucleus (Figure 1.2A). In this manner, the level of p27xic expression in the progenitor cells can indirectly affect the decision to proliferate or differentiate by controlling G1 length. But what controls the level of p27xic? Recent work has demonstrated that p27xic is an aPKC substrate and phosphorylation regulates its ability to inhibit the G1 to S transition. Phosphorylation prevents p27xic's binding to Cdk2 providing a simple, but elegant method for coupling aPKC activity to cell cycle control, and ultimately the decision to proliferate or differentiate (Sabherwal et al., 2014) (Figure 1.2B).

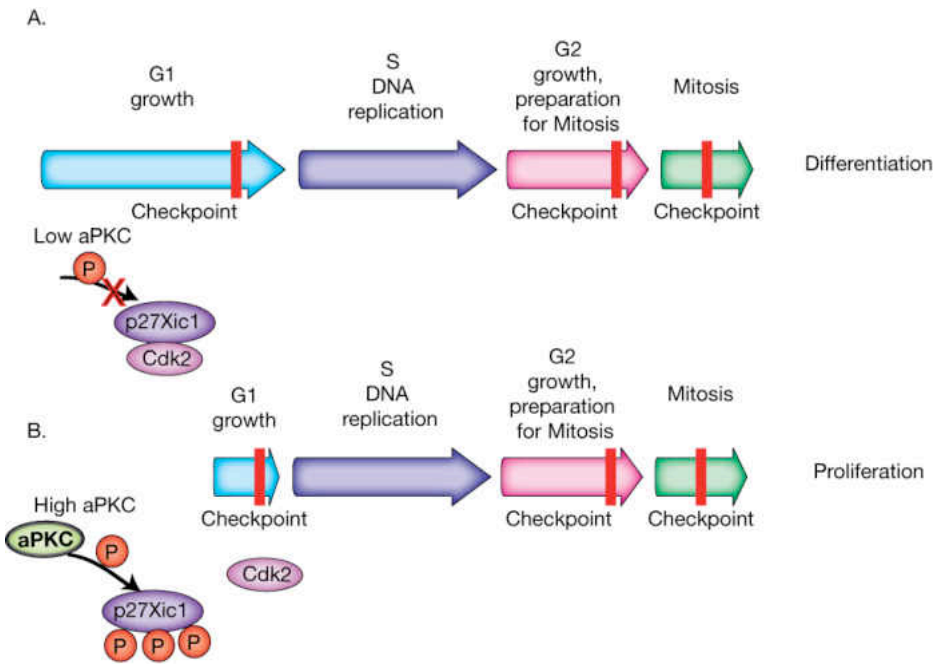


Figure 1.2. aPKC regulation of the cell cycle. (A) When aPKC levels are low, p27Xic1 is able to elongate the G1 to S transition by binding to Cdk2, which can lead to differentiation in *Xenopus* neuroectoderm progenitor cells. (B) When aPKC levels are high, p27Xic1 phosphorylation by aPKC blocks p27Xic1 binding of Cdk2, shortening the G1 to S transition to promote proliferation.

Transcriptional programming by aPKC

Hedgehog (HH) signaling is important for cell fate decisions that specify the animal body plan (Briscoe and Thérond, 2013). In the absence of HH ligand, Patched (Ptch) represses HH signaling through inhibition of the receptor Smoothened (Smo) (Varjosalo and Taipale, 2008) (Figure 1.3). When HH binds Ptch at the membrane, transcriptional activators such as GLI (Cubitus interruptus in *Drosophila*) become active (Varjosalo and Taipale, 2008). This pathway can regulate stem cell proliferation versus differentiation decisions (Brownell et al., 2011; Ruiz i Altaba et al., 2002) and is often reactivated during the initiation and progression of cancers such as basal cell carcinomas (BCCs) and lung squamous cell carcinomas (LSCCs) (Ng and Curran, 2011; Rubin et al., 2005). Binding to Ptch requires numerous HH post-translational modifications including specific proteolysis followed by palmitoylation by HH acyl transferase (HHAT) (Varjosalo and Taipale, 2008). Once HH ligand binds to Ptch, Ptch no longer inhibits Smo, resulting in translocation of GLI to the nucleus and subsequent activation of proliferative genes(Briscoe and Thérond, 2013). Recently, aPKC has been found to regulate multiple points within the HH pathway. Activity of aPKC leads to upregulation of the HH ligand, phosphorylation of the receptor Smoothened, and activation of the bifunctional transcriptional regulator of HH signaling, GLI (Atwood et al., 2013; Jiang et al., 2014; Justilien et al., 2014) (Figure 1.3).

There are several mechanisms by which aPKC regulates HH signaling. First, expression of the HHAT enzyme is dependent on aPKC activity. This control occurs by aPKC's phosphorylation of SOX2, an important transcriptional regulator of stem cell

maintenance. SOX2 modification by aPKC allows it to bind the HHAT promoter region (Justilien et al., 2014) (Figure 1.3). This leads to an increase in functional HH ligands. Upregulation of HHAT by aPKC can be important for tumorigenic growth by maintaining stemness, as has been demonstrated for LSCC oncospheres (Justilien et al., 2014).

HH signaling can also be regulated by aPKC downstream of the Ptch receptor. The GLI1 transcription factor is an aPKC substrate (Atwood et al., 2013), and, as with SOX2, phosphorylation activates transcription of GLI1 target genes including aPKC itself (Figure 1.3). This positive feedback loop can lead to the development and progression of basal cell carcinomas (BCCs) independent of Smo activation of GLI1 (Atwood et al., 2013) (Figure 1.3). Currently, Smo inhibitors are used to treat BCCs but the tumors can develop resistance (Atwood et al., 2015; Briscoe and Thérond, 2013). Inhibition of aPKC signaling inhibits BCC tumor-growth indicating that inhibitors could have therapeutic potential for treating BCCs (Atwood et al., 2015). In *Drosophila,* aPKC phosphorylates Smo and GLI (Cubitus interruptus in *Drosophila*) to polarize them basolaterally, thereby promoting HH signaling during early wing development (Jiang et al., 2014). However, the molecular mechanism by which aPKC activity is controlled during HH signaling remains unclear.

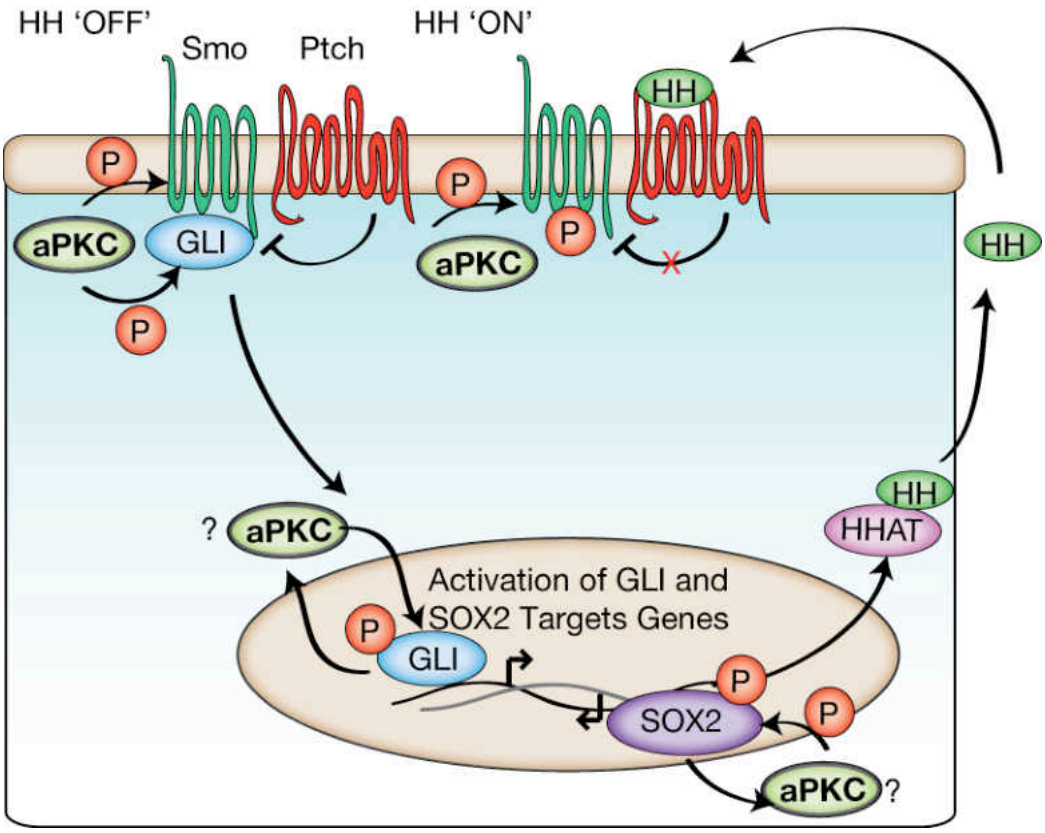


Figure 1.3. aPKC regulation of Hedgehog signaling. In basal cell carcinomas (BCCs) and lung squamous cell carcinomas (LSCCs) aPKC is able to phosphorylate GLI (BCCs) and SOX2 (LSCCs) transcription factors. These phosphorylations can lead to positive feedback, upregulating HH signaling genes including HHAT and aPKC itself. This activation can occur independently of HH ligand receptor binding. In the *Drosophlia* developing wing, aPKC phosphorylates the Smoothened receptor to regulate its activity and its subsequent proper development.

aPKC regulation of Wnt signaling

Many tissues, such as the epidermal and intestinal epithelia, undergo rapid turnover requiring constant differentiation from precursor cells for tissue maintenance. In mammalian epidermal models, aPKC regulates cell fate by ensuring proper division orientation (Niessen et al., 2013). Adult intestinal stem cells are continually replenishing the cells of the epithelium, which is turned over ever 3-5 days (Barker, 2014; Jiang and Edgar, 2011). In these adult stem cell models, precise regulation of β-Catenin (Wnt

signaling) and Yap (Hippo pathway) is required for maintenance of tissue homeostasis and prevention of tumor initiation and progression (Azzolin et al., 2014; Clevers et al., 2014).

In the absence of Wnt ligands, β-Catenin is degraded by the "destruction" complex composed of the tumor suppressor adenomatous polyposis coli (APC), scaffolding protein Axin, glycogen synthase-3 (GSK-3β) and casein kinase 1 (CK1). While the complex is intact, β-Catenin is phosphorylated by GSK-3β and degraded by the proteasome (Stamos and Weis, 2013). In the absence of nuclear β-Catenin, downstream Wnt-dependent target genes are not transcribed, inhibiting proliferative and growth signals (Figure 1.4A). When Wnt is bound to the receptor Frizzled (FZD) and a co-receptor, Axin is thought to be degraded and the destruction complex dissociates, concomitantly stabilizing β-Catenin levels, allowing for nuclear translocation and binding to co-activator TCF/LEF proteins. Ultimately, this leads to the transcription of Wnt-dependent target genes (Stamos and Weis, 2013) (Figure 1.4B). Wnt signaling has been implicated in polarity through interactions with the Par complex in migratory cells (Etienne-Manneville and Hall, 2003). Recent work has shed light on how aPKC might be playing a direct roll in Wnt signaling.

aPKC has now been identified as a component of destruction complex that interacts with Yap and β-Catenin (Llado et al., 2015). While best known for their role in Hippo pathway signaling (Yu and Guan, 2013), Yap and Yaz also interact with the destruction complex (Azzolin et al., 2012, 2014). aPKC phosphorylates both β-Catenin and Yap, preventing their nuclear accumulation, thereby inhibiting Wnt and Hippo downstream targets required for proliferation and cell growth (Llado et al., 2015) (Figure

1.4). β-Catenin must be phosphorylated at its aPKC phosphorylation site (either by aPKC or another kinase) before GSK-3β can act on it (Amit et al., 2002; Liu et al., 2002). Yap activity is increased by aPKC, in a manner that is at least partially independent from canonical Hippo signaling. In *Drosophila,* GSK3b regulates polarity by phosphorylating aPKC, which targets it for proteasomal degradation (Colosimo et al., 2010) suggesting crosstalk between these pathways.

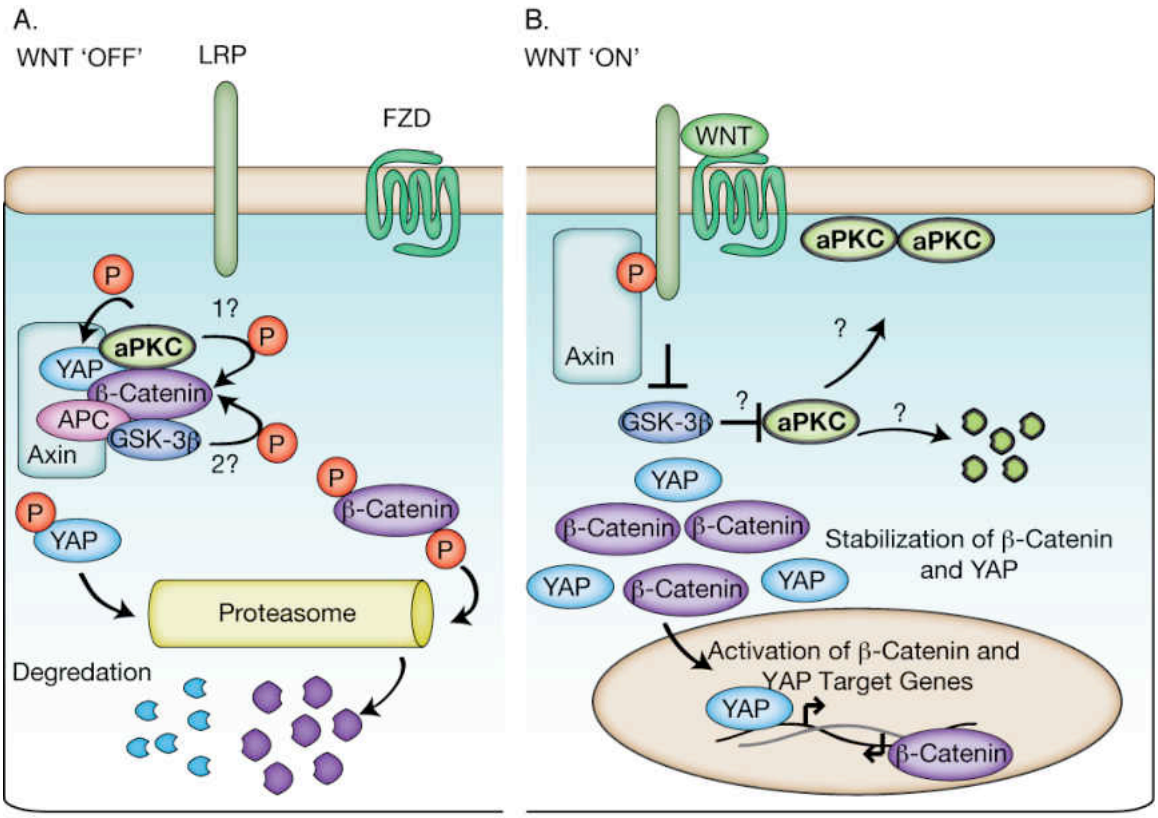


Figure 1.4. aPKC regulation of Wnt signaling. (A) aPKC is part of the destruction complex, where it can phosphorylate (1) β-Catenin to prime it for (2) GSK-3β phosphorylation and subsequent proteasomal degradation. aPKC is also able to phosphorylate YAP, leading to proteasomal degradation. (B) Loss of aPKC or Wnt binding leads to disassembly of the destruction complex and activation of Wnt signaling favoring a proliferative state. The fate of aPKC once the destruction complex is inactivated is unknown.

aPKC regulation of JAK/Stat

Janus Kinase (JAK) and Signal transducer and activator of transcription (Stat) are important growth regulators that play a prominent role in development and tumor progression (Kortylewski et al., 2005; Yu et al., 2009; Al Zaid Siddiquee and Turkson, 2008). Numerous signaling pathways activate JAK/Stat by inducing JAK recruitment to Stat and subsequent Stat phosphorylation. During IL6 cytokine activation, phosphorylation of the Stat3 isoform by JAK leads to Stat3 nuclear translocation where it activates proliferation and survival genes and represses differentiation genes (Yu et al., 2014). Stat3 has also been implicated in the maintenance of cancer stem cells (CSCs) (Magee et al., 2012; Visvader and Lindeman, 2008).

In a recent study, aPKC activity was found to activate Stat3 in a mammalian model of breast cancer (Guyer and Macara, 2015). Activation occurs via aPKC's interaction with the NF-**k**b signaling pathway, which is up regulated in many human cancers (Bassères and Baldwin, 2006). In this system, aPKC becomes active in the cytoplasm after loss of polarity where it activates IKKβ, ultimately causing increased IL6 production (Duran et al., 2003; Win and Acevedo-Duncan, 2008). This leads to a positive feedback loop associated with proliferation and tumor progression (Figure 1.5). Up-regulation of IL6 by active aPKC in unpolarized cells also occurs in *Drosophila* models that combine polarity loss with oncogenic transformations (Bunker et al., 2015). In fact, constitutively active aPKC is sufficient to induce IL6 (Upd in *Drosophila*) expression, although the effect is dependent on the *Drosophila* ortholog of YAP (Yki) (Bunker et al., 2015). Whether or not aPKC induces IL6 through YAP via the canonical Hippo pathway

signaling or as part of the destruction complex (i.e. Wnt signaling), remains to be resolved.

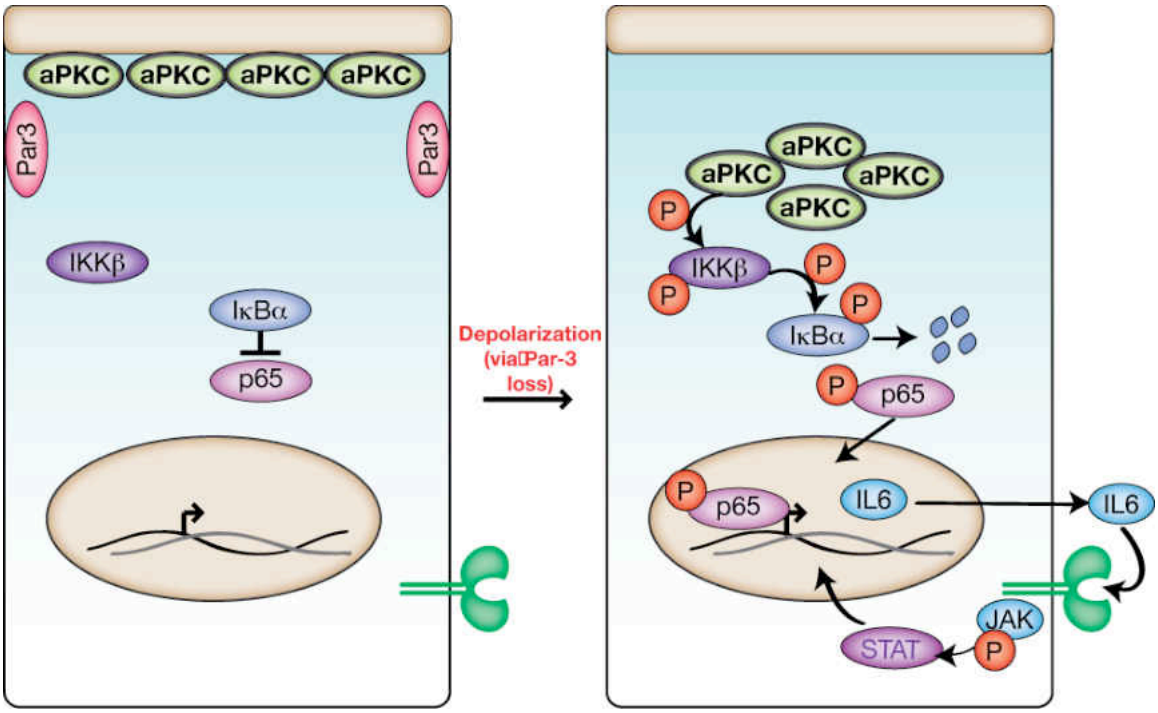


Figure 1.5. aPKC regulation of JAK/Stat signaling. Loss of polarity leads to cytoplasmic aPKC which causes activation of IKKβ, degradation of IκBα, and translocation of p65 to the nucleus to upregulate IL6 production. The increase in IL6 leads to a positive feedback loop with JAK/Stat3 signaling, which, when unregulated, leads to proliferation and tumor progression in a breast cancer model.

Concluding remarks

How cellular diversity is generated during development is one of the most fundamental questions in Biology. Once development is complete, homeostasis requires the constant activity of progenitor cells to replenish rapidly turned over differentiated products. Each of these processes is highly intertwined with proliferation pathways, such that defects are commonly associated with tumorigenesis. Our understanding of the molecular mechanisms that control cell fate decisions is still in its infancy, but it is now clear that the atypical members of the PKC kinase family are involved in many aspects of

fate specification. Some of these functions relate to aPKC's activity in regulating cell polarity, but there are newly identified polarity-independent aPKC functions (both in normal and pathological Biology) that are essential for conferring proper cell identity. We expect that many more aPKC substrates and regulatory proteins remain to be found, and that fitting them into the puzzle of cell fate will help provide a more complete picture of this fundamental process.

QUESTIONS STILL REMAINING IN CELL POLARITY AND REGULATION OF APKC:

-How is aPKC activated during ACD of Drosophila neuroblasts?

-How does aPKC become polarized?

-What are the roles of each of the aPKC domains in this process?

-Are aPKC localization and activation coupled during ACD of Drosophila neuroblast?

While many of the proteins required for cell polarization have been identified, the mechanism by which polarization occurs is still being elucidated. Much of what is currently known comes from the use of *Drosophila* as a model organism. In order to attempt to answer the above-mentioned outstanding questions, I used a combination of genetic and biochemical approaches to define the mechanisms that are involved in the activation and localization of aPKC.

BRIDGE TO CHAPTER II

In the previous chapter I introduced the importance of the protein aPKC in the generation of cellular polarity, as well as its importance in regulation of the cell cycle and

transcription factors, all of which govern cell fate decisions. Also, I introduced the *Drosophila* neuroblast as an important model system to investigate the proteins necessary to generate polarity and subsequently, the current understanding of the mechanisms by which polarity is established. In the next chapter, I seek to answer an outstanding question when it comes to the activation of aPKC by the upstream kinase PDK1. Biochemical approaches have revealed that PDK1 phosphorylates the activation loop of aPKC, which is then autophosphorylated at the aPKC turn motif. It is generally, but not universally, accepted that this phosphorylation event is required for aPKC activity. Recent crystal structures have even demonstrated that a mammalian aPKC kinase domain adopts an active conformation without activation loop phosphorylation. Even less clear, is if the observed activity differences between aPKC phosphorylated and unphosphorylated at the activation loop, is physiologically relevant. In the next chapter, I carefully investigated the importance of activation loop phosphorylation with a highly quantitative, *in vitro* approach. I then explain my work using the *Drosophila* neuroblast as model, demonstrating that phosphorylation of aPKC activation loop and turn motif are required for both, catalytic activity, and spatial regulation during asymmetric cell division.

CHAPTER II

REGULATION OF NEUROBLAST POLARITY AND SELF-RENEWAL BY PHOSPHOINOSITIDE DEPENDENT KINASE 1

This chapter contains unpublished co-authored material

Author contributions: Michael Drummond, Chiharu Graybill, and Kenneth Prehoda designed the research; Michael Drummond and Chiharu Graybill performed the research; Michael Drummond, Chiharu Graybill, and Kenneth Prehoda analyzed the data; and Michael Drummond, Chiharu Graybill, and Kenneth Prehoda wrote the manuscript.

INTRODUCTION

The generation and maintenance of molecularly distinct cortical domains is necessary for a wide range of cellular functions. In many animal cells, cortical polarity is regulated by the Par complex, which consists of the proteins Par-3, Par-6, and atypical Protein Kinase C (aPKC) (Goldstein and Macara, 2007; Prehoda, 2009). In cells such as epithelia, or neural stem cells, the Par complex is confined to a specific cortical domain where it excludes cell-type specific proteins such as the tumor suppressor Lethal giant larvae (Lgl) and the neuronal fate determinant Miranda (Doe, 2008; Tepass, 2012). Cortical exclusion is established and maintained by aPKC, as Par polarized factors are substrates that are phosphorylated upon entering the Par cortical domain. Substrate phosphorylation inhibits cortical association, ensuring that Par polarized proteins don't

accumulate in the Par domain. Because of the central role of aPKC in Par-mediated polarity, slight perturbations in aPKC activity can have severe consequences, such as uncontrolled proliferation and metastatic tumor formation (Bunker et al., 2015; Lee et al., 2006a, 2006b; Parker et al., 2014). Although aPKC activity must by tightly regulated both spatially and temporally during cell polarization, the mechanisms that control aPKC are poorly understood. Here we have examined the regulation of aPKC from the perspective of its membership in the larger family of PKC enzymes.

The PKC family of protein kinases includes conventional and novel, in addition to atypical subfamilies (1 isozymes in flies, 2 in humans). Individual PKC family members regulate diverse cellular processes, from proliferation to apoptosis (Antal and Newton, 2014; Breitkreutz et al., 2007; Newton, 2010). A key difference between atypical PKCs is that they are not activated by diacylglycerol (DAG), unlike their conventional and novel counterparts (Colón-González and Kazanietz, 2006; Kazanietz et al., 1994). This difference arises from a unique regulatory motif architecture as aPKCs lack the DAG-binding C2 domain (Newton, 2010). Besides these architectural differences, however, all family members share a number of key regulatory features. For example, each contains a "pseudosubstrate" motif that competes with substrates for active site binding. Par-6 binding to aPKC has been shown to displace its pseudosubstrate from the kinase domain, allowing it to phosphorylate substrates such as Lgl (Graybill et al., 2012). Additionally, PKC catalytic domains have several sites that are phosphorylated and these post-translational modifications can be critical for catalytic activity, although much less is known about their involvement in spatial regulation (Newton, 2010).

In order to identify genes responsible for regulating aPKC activity and localization during polarization, we performed a candidate based RNAi screen to uncover genes that perturb self-renewal of *Drosophilia* neural stem cells (neuroblasts). During development, neuroblasts undergo repeated asymmetric cell divisions to generate the central nervous system. Early in mitosis, neuroblasts polarize the Par complex to the apical cortex where it confines neuronal fate determinants to the basal cortex. Mitotic polarity leads to one daughter cell that retains the neuroblast fate (i.e. self renews), and another that differentiates. Thus, proper regulation of aPKC is critical for maintenance of the neuroblast pool through self-renewal at each division and this phenomenon can be exploited to identify factors that control aPKC activity.

MATERIALS AND METHODS

Purification of aPKC variants

HEK293 F cells (1 x 10^6 cells/mL) were transfected with aPKC (pCMV His_6-aPKC 1-606, pCMV His_6-aPKC 1-606 K293W, pCMV His_6-aPKC 259-606, pCMV His_6-aPKC 259-606 T422A, or pCMV His_6-aPKC 259-606 T574A) and pCMV dPDK-1 (without any tag) using the 293fectin transfection reagent (Life Technology). The cells were incubated at 37°C for 48-72 hours. To harvest, the cells were resuspended with Ni^{2+} lysis buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 1 mM MgCl, 10 mM β-ME, 10 mM Imidazole, adjusted to pH 7.5 with NaOH). The cells were lysed by passage through a 21-gauge needle and the lysate was clarified by centrifugation at 15,000 rpm for 30 minutes at 4°C. The supernatant was incubated with Ni^{2+} NTA resins for 45 minutes at 4°C followed by washing with lysis buffer. Following elution using Ni^{2+} elution buffer

(lysis buffer with 250 mM Imidazole), the eluted proteins were dialyzed at 4°C for 4 hours against 20 mM Tris-HCl, 50 mM NaCl, 1 mM DTT. The concentration of aPKC was determined by comparing its reactivity with an anti-aPKC antibody (Santa Cruz Biotech) with that of a standard of known concentration (bacterially expressed aPKC kinase domain purified and quantified using a Bradford dye binding assay) on a western blot.

***In vitro* kinase activity assay**

aPKC kinase activity was measured as previously described (Graybill et al., 2012). Briefly, the purified aPKC kinase domain was diluted to concentrations at which the incorporation of radiolabeled phosphate from [γ^{-32}P]ATP into MBP-Lgl peptides were linear with respect to time and the enzyme concentrations. The diluted enzymes were preincubated in the assay buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$) with a wide range of MBP-Lgl peptide concentrations at 30°C for 5 minutes. The reactions were initiated by adding 1mM ATP spiked with [γ^{-32}P]ATP (~1.0 x 10^6/nmol ATP). The reactions were incubated at 30°C for 10 minutes. The reaction mixtures were blotted on Grade P81 phosphopaper (Whatman). The reactions were quenched by immediately submerging the blotted P81 paper in 75 mM H_3PO_4. 5 mL of scintillation fluid were added to measure the radioactive decays by liquid scintillation counter. The kinetic parameters were calculated by fitting the data to the Michealis-Menten equation in GraphPad Prism.

Antibodies and immunofluoresence

Immunoflourescence was performed as previously described (Atwood and Prehoda, 2009). In brief, third instar larval brains were dissected and fixed in PBS with 4% paraformaldhyde for 20 minutes. After being fixed, brains were washed with PBS containing 0.1% TX-100 (PBS-T) 3 times (quick rinse) followed by a single 20 minute wash. Next, the brains were blocked in PBS with 1% BSA and 0.1 TX-100 (PBS-BT) for 30 minutes and then incubated with primary antibodies dilluted in PBS-BT overnight at 4°C on a nutator. Primary antibodies were removed and brains were washed 3 times with PBS-BT for 15 minutes each. Following washes, brains were incubated in secondary antibodies diluted in PBS-BT for 2 hours at room-temperature in the absence of light. Finally, brains were wash 3 times with PBS-T for 15 minutes each and placed in Vectashield H-1000 prior to mounting on slides.

Primary antibodies for this study included: rabbit anti-aPKCζ (1:1000; C-20, Santa Cruz Biotechnology), rabbit anti-p-aPKCζ (Thr 410) (1:1000; sc-12894-R, Santa Cruz Biotechnology), rabbit anti-p-aPKCζ (T560) (1:1000; EP2037AY, Abcam), guinea pig anti-Baz (1:1000; Doe lab), Chicken anti-GFP (1:5000; ab13970, Abcam) mouse anti-HA (1:500; Covance), guinea pig anti-Miranda and Rat anti-Miranda (1:500; Doe lab), and rat anti-Par6 (1:1000; in house). Secondary antibodies were donkey anti-guinea pig 405 and donkey anti-rat 405 (1:500; Jackson Immuno-Research Laboratories) donkey anti-mouse 488 and donkey anti-chicken 488 (1:500; Jackson Immuno-Research Laboratories), goat anti-rat 555 (1:500; Invitrogen), donkey anti-rabit 649 and donkey anti-rat 649 (1:250; Jackson Immuno-Research Laboratories).

Confocal images were collected using a Zeiss 700 equipped with a 40x oil immersion objective/1.43 NA or an Olympus Fluorview 1000 equipped with a 40x oil immersion objective/1.30 NA. Figures were generated using Fiji, Adobe Photoshop, and Adobe Illustrator. Quantifications were performed using Graphpad Prism.

Fly strains and genetics

We used w^{1118} as our wild-type strain. In order to generate aPKC FL, aPKC K293W, aPKC T422A, and aPKC T574A expressing flies, the coding sequence of each was subcloned into a PUAST-attb plasmid containing a 5' Hemagglutinin (HA) and transformants were created and isolated by Genetic Services using standard techniques. Attp2, on chromosome 3R was the chosen landing site for our transgenics. For overexpressions, we used *Inscuteable-GAL4* at 30°C and dissected larvae when they became wandering third instar larvae (approximately 76 ALH at 30°C). RNAi was performed using *Worniu-GAL4, UAS-DCR2* (Doe Lab) crossed to either PDK1 RNAi line (Bloomington stock number 27725 and VDRC 109812) at 30°C and dissected when their wild type counterparts were wandering third instar larvae.

MARCM clones were generated as previously described (Lee and Luo, 1999). In brief, $FRT^{G13}aPKC^{k06403}/Cyo$ virgins were crossed to *;;UAS-aPKC FL*, *;;UAS-HA:aPKC T422A*, *;;UAS-HA:aPKC T574A,* or *;;UAS-HA:aPKC K293W* male flies between 3 and 9 days old. F1s males lacking Cyo were collected and crossed to virgin *elav-GAL4, UAS-mCD8:GFP, hs:flp ;* FRT^{G13}*, tubPGal80* (Doe Lab). Larvae were heat shocked 24 hours after larval hatching at 37°C for 90 minutes and again 10-16 hours after the first heat shock. Larvae were allowed to grow to the wandering third instar larval stage at 22°C or

25°C and then dissected and immunostained. aPKC null clones were positively marked with GFP and overexpressed aPKC variants were detected using mouse anti-HA.

RESULTS

Identification of PDK1 as a regulator of neuroblast self-renewal

To identify factors that regulate aPKC activity during *Drosophila* neuroblast asymmetric cell division, we screened a panel of UAS-driven RNAi's directed against putative PKC family regulators for their effect on neuroblast self renewal. In the larval central nervous system, each brain lobe normally contains approximately 100 central brain neuroblasts, as assessed by the markers Deadpan (Dpn) and Mira (Figure 2.1A), but defects in self-renewal can dramatically reduce the neuroblast population (Doe, 2008; Homem and Knoblich, 2012). We found that expression of an RNAi directed against *phosphoinositide dependent kinase 1 (pdk1)* resulted in a significant decrease in the number of larval central brain neuroblasts, and we observed a similar effect with an RNA targeted to a different region of the *pdk1* gene (Figure 2.1B-D). Apoptosis can also lead to a reduction in neuroblast number, but we found that expression of the apoptotic inhibitor p35 did not rescue the effects of *pdk1* RNAi (Figure 2.1D)

PDK1 acts upstream of PKC family members by phosphorylating their activation loop segment (residue T422 in *Drosophila* aPKC), a modification that can be a prerequisite for activity (Chou et al., 1998; Dutil et al., 1998; Le Good et al., 1998; Standaert et al., 1997, 2001). However, there is conflicting evidence for the role of activation loop phosphorylation in aPKC activity (Ranganathan et al., 2007; Wang et al., 2012). The kinase domain's turn motif (T574) can also be phosphorylated, which has

been though to be an autocatalytic event, although recent evidence suggest that this may not be case (Cameron et al., 2009; Le Good et al., 1998) . We stained neuroblasts expressing *pdk1* RNAi with an antibody specific to T422 phosphorylated aPKC (anti pT410) and found that the apical cortical signal was significantly reduced compared to wild-type cells. Surprisingly, we also observed a large reduction in Par-6 apical staining in the *pdk1* RNAi neuroblasts, indicating that PDK1 is required for Par complex targeting to the apical cortex (Figure 2.1F-H). Miranda's polarization was largely unaffected by PDK1 knockdown indicating that sufficient polarized aPKC activity remained to prevent Miranda from entering the Par cortical domain (Figure 2.1F-H). Although we were unable to visualize endogenous PDK1, an ectopically expressed, tagged version of the protein localized throughout the neuroblast cytoplasm, with possible cortical enrichment (Figure 2.1I).

Active loop and turn motif phosphorylation are required for full *Drosophila* aPKC kinase activity *in vitro*

The effect of PDK1 knockdown could result from the reduction in phosphorylated aPKC activation segment, or from the loss of some other PDK1 activity (e.g. phosphorylation of another PKC family member or other substrate). To determine which aspects of the *pdk1* phenotype arise from direct phosphorylation of aPKC, we generated non-phosphorylatable aPKC variants – one for the activation segment (T422A) and one for the turn motif (T574A) – and measured their activity both *in vitro* and *in vivo* (Figure 2.2A). The importance of kinase domain phosphorylations for aPKC's catalytic activity has been unclear because of conflicting *in vitro* and structural data, which demonstrated

that substrate binding to a kinase domain that lacked the modifications. To help resolve this controversy, we used a quantitative approach measuring activity over a wide range of substrate (a peptide from Lethal giant larvae) concentrations. This allowed us to avoid artifacts associated with single substrate concentration assays and to detect differences in both Michaelis-Menten kinetic parameters, K_M and k_{cat}. We purified *Drosophila* aPKC kinase domains (residues 259-606; the full length protein is autoinhibited) containing the T422A or T574A mutations and one with a "kinase-dead" K293W mutation, which prevents ATP-binding, in addition to the wild-type protein. When compared to the activity of wild-type aPKC, both the T422A and T574A variants had reduced catalytic activity (Figure 2.2B). Both mutations caused K_M to increase, consistent with a role for the phosphorylations in substrate binding, but k_{cat} was most affected indicating that they serve a critical function in the catalytic step (Figure 2.2C). Thus, we find that modification of aPKC's kinase domain is required for full catalytic activity, but that significant residual activity remains even in the absence of these phosphorylations.

Kinase domain phosphorylations are required for aPKC activity and polarization in neuroblasts

Although the unphosphorylated aPKC variants have residual catalytic activity, we sought to determine if this level of activity is sufficient to mediate function *in vivo*. We generated transgenic *Drosophila* lines containing HA-tagged WT, T422A, or T574A aPKCs under the control of the *UAS* promoter and drove their expression using *Inscutable-Gal4*, which is specific for the neuroblasts of the larval brain. Expression of

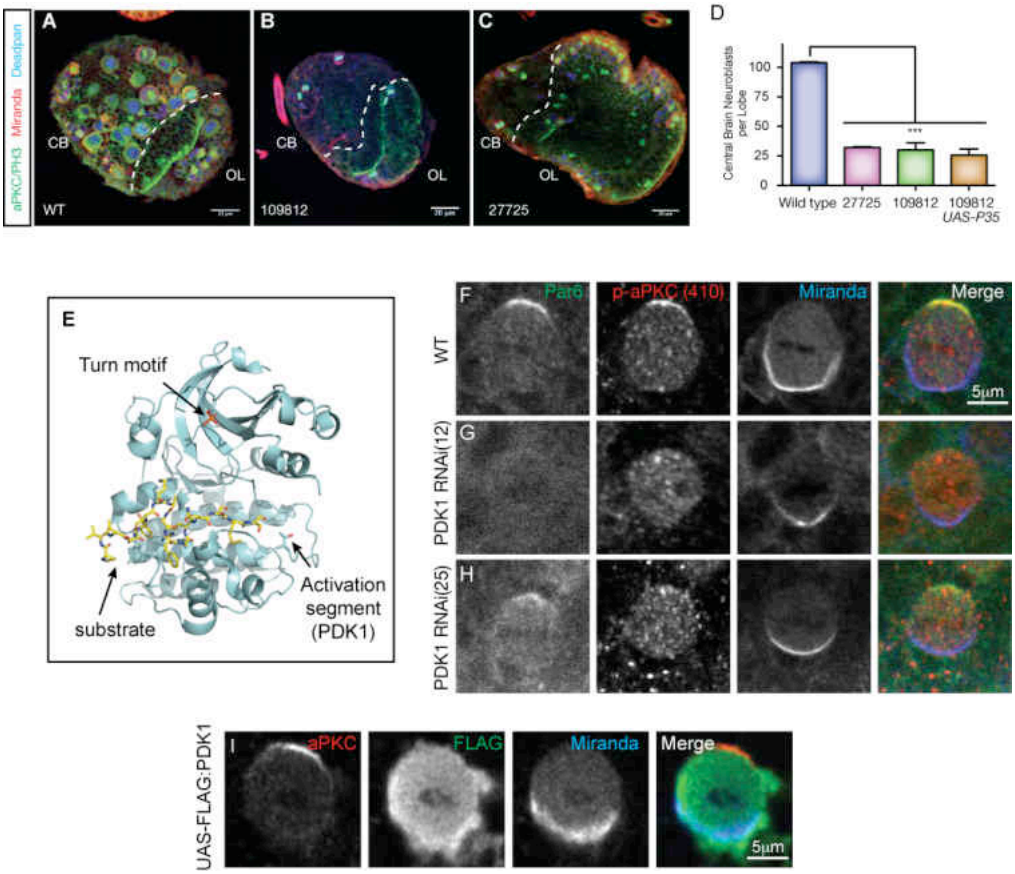
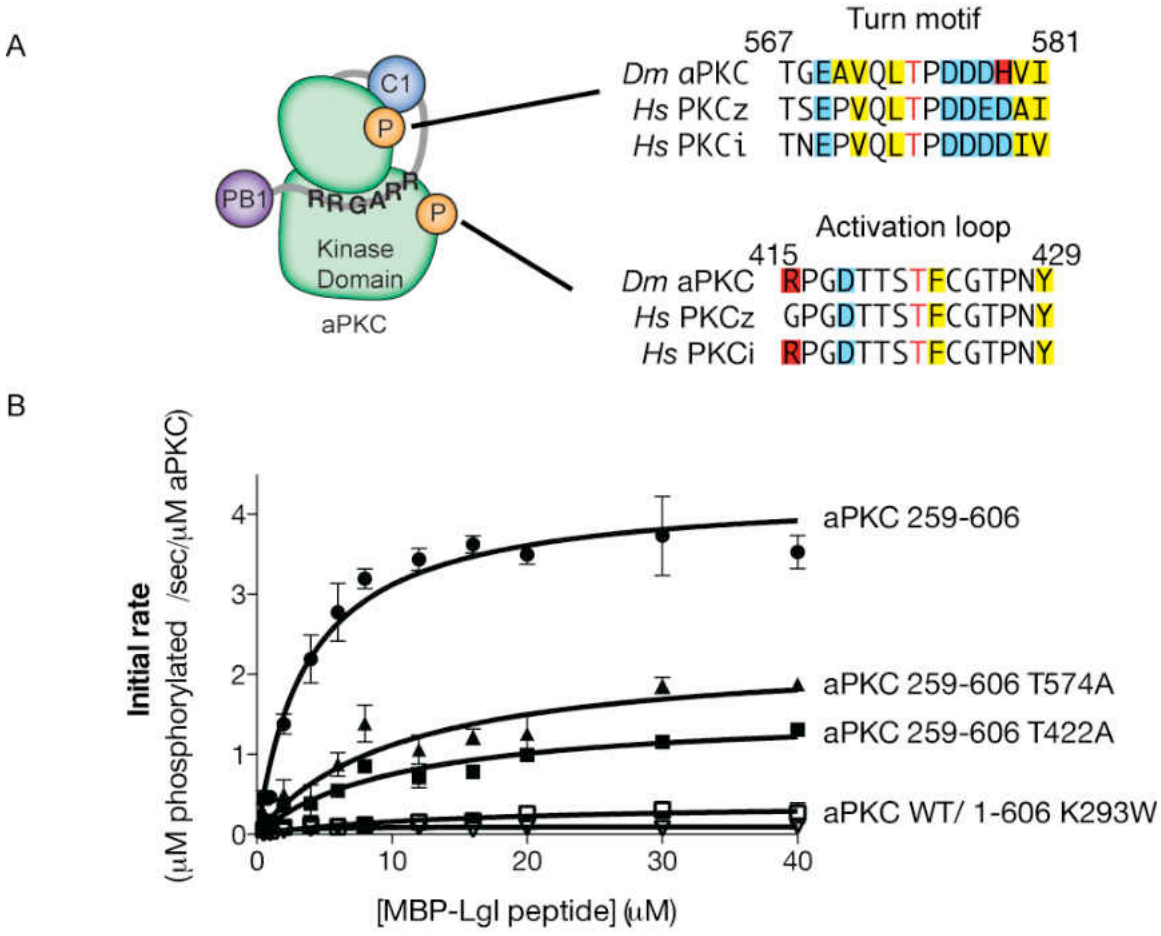


Figure 2.1. Loss of PDK1 impairs neuroblast self-renewal and proper aPKC activation loop phosphorylation. (A-C) Representative z-slice of either wild type or PDK1 RNAi expressing brain lobes showing the central brain (CB) and the optic lobe (OL) stained with aPKC/PH3, Miranda, and Deadpan. RNAi was driven using *worniu-GAL4, UAS-DCR2* at 30°C until wild type counterparts reached the wandering third instar larval stage. Scale bars represents 20 μM (A) Wild type average central brain neuroblasts per lobe 103.83 ± 2.04 sd. (B) PDK1 RNAi (27725) average central brain neuroblasts per lobe 32.16 ± 1.47 sd. (C) PDK1 RNAi (109812) average central brain neuroblast per lobe 30 ± 15.19 sd. (D) Quantification of total central brain neuroblast for six lobes per condition with the orange column representing PDK1 RNAi (109812) with expression of P35 to block apoptosis, average 25.67 +/1 5.24 sd. *** Represents a P-value < 0.0001. Mean and SEM are plotted. (E) Ribbon diagram representing the crystal structure of PKCι kinase domain bound to a Par-3 peptide (PDB 4DC2). The peptide binding, activation loop, and turn motif are shown. (F-H) Wild type or PDK1 RNAi expressing neuroblasts labeled with Par-6, p-410 aPKC (activation loop specific antibody), and Miranda. (F) Wild type showing proper apico-basal polarity. (G) PDK1 RNAi (27725) showing reduced Par-6 and activation loop labeling, but maintained Miranda polarization. (H) PDK1 RNAi (109812) showing reduced Par-6 and activation loop labeling, but maintained Miranda polarization. (J) Overexpression of a FLAG-tagged PDK1 demonstrating the cytoplasmic with possible cortical localization of PDK1.



C

aPKC variants	K_M (μM)	k_{cat} (sec^{-1})
Wild type	1.7 ± 1.0	0.10 ± 0.01
1-606 K293W	15.3 ± 9.6	0.4 ± 0.1
259-606	3.8 ± 0.6	4.3 ± 0.2
259-606 T422A	10.5 ± 2.4	1.5 ± 0.1
259-606 T574A	10.4 ± 3.3	2.3 ± 0.3

Figure 2.2. The effects of mutation of the activation loop or the turn motif on aPKC catalytic activity. (A) Domain architecture of aPKC with amino acid sequence alignments of activation loop and turn motif regions containing the phospho-accepting Thr residues (highlighted in red). The numbering is based on Drosophila aPKC. (PB1 Phox/Bem1 domain; PS = pseudosubstrate; C1 is a cysteine rich domains). (B) Kinetic analyses of aPKC activity, showing that the mutation from Thr to Ala in either the activation loop or the turn motif resulted in increased K_M and reduced k_{cat}. (C) The summary values of K_M and k_{cat} of aPKC variants used in kinetic analyses.

WT aPKC had no detectable effect on neuroblast polarity or aPKC localization (Figure 2.3B). Slight perturbations in aPKC activity can alter larval brain neuroblast number, however we found that the neuroblast count from Insc-Gal4>*WT aPKC FL* (data not shown) animals were normal. The ability of neuroblasts to accommodate increased aPKC expression suggests that the regulatory pathways that control its activity are robust enough to maintain aPKC activity within an appropriate functional range. We also assessed Insc-Gal4>*aPKC* function by rescuing $aPKC^{k06403}$ positively marked mutant clones. In $aPKC^{k06403}$ clones, the lack of apical aPKC activity causes Miranda to become depolarized. As seen in Figure 2.3, transgene expressed aPKC localized apically in mutant clones and restored Miranda polarity by confining it to the basal cortex indicating that transgene expressed aPKC is functional in this context.

We first examined the role of the T422 phosphorylation in neuroblast polarity. Neuroblasts expressing both endogenous and the non-phosphorylatable T422A aPKC variant had unperturbed Miranda localization (Figure 2.3C), indicating that basal polarity was unaffected. Surprisingly, however, aPKC T422A was no longer restricted to the apical cortex, localizing around the entire cortex including colocalizing at the basal domain with Miranda (Figure 2.3C). The colocalization with Miranda indicates that the protein is inactive, either because the residual catalytic activity of aPKC T422A observed *in vitro* is insufficient to displace Miranda from the cortex, or the protein is autoinhibited. Next, we examined the phosphorylation state of the activation loop and turn motif in these cells. Neuroblasts expressing aPKC T422A had limited cortical pT422 signal (Figure 2.3C). While this is consistent with the inability of T422A aPKC to be phosphorylated at this site, the reduced apical staining with this antibody indicates that

the endogenous protein is also affected. The phosphorylated turn motif antibody (pT574) yielded a depolarized cortical signal similar to the HA staining for the transgenic protein (Figure 2.3C'). We interpret these results in the following manner: the reduction in pT422 signal at the apical cortex is a consequence of aPKC T422A competition with endogenous aPKC, although sufficient endogenous aPKC remains to exclude Miranda and phosphorylate T574 on both the wild type and T422A proteins. We also examined aPKC T422A in $aPKC^{k06403}$ clones to determine if it could rescue polarity loss, and to ensure that the presence of wild-type aPKC does not influence the transgenically expressed protein's behavior. We found that aPKC T422A was unable to rescue the loss of Miranda polarity in $aPKC^{k06403}$ clones (Figure 2.3I). Furthermore, aPKC T422A localized throughout the neuroblast cortex, as we observed in the overexpression context. These results indicate that T422 phosphorylation is required for aPKC activity, either because of its role in catalytic activity or a possible role in activation of the autoinhibited conformation. Additionally, phosphorylation at this site is necessary for aPKC polarity by preventing its localization at the basal cortex.

Next, we examined the role of turn motif T574 phosphorylation in neuroblast polarity. Phosphorylation at this site was thought to occur autocatalytically *in trans* following activation segment phosphorylation by PDK1, although recent evidence suggests that other kinases (e.g. mTOR) may directly modify the turn motif (Ikenoue et al., 2008; Li and Gao, 2014). Similar to the unphosporylatable activation segment aPKC variant, aPKC T574A expressed alongside endogenous aPKC localized around the entire

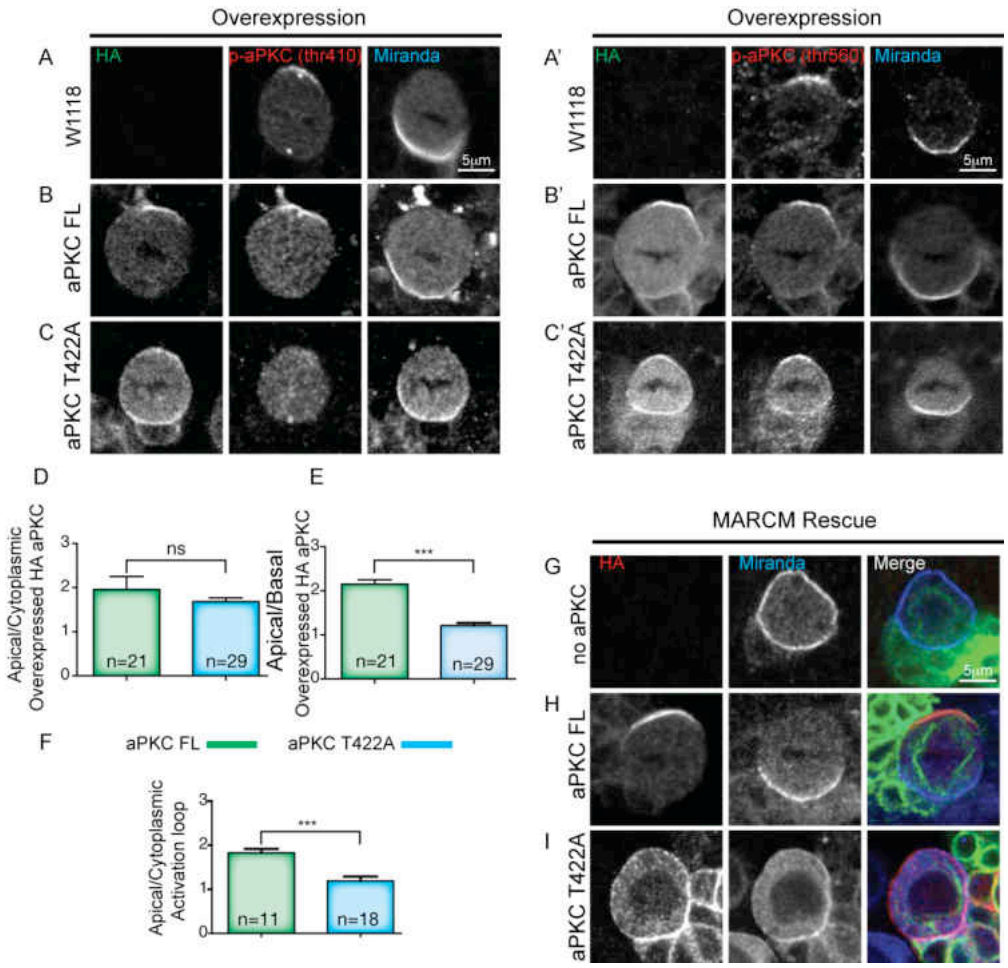


Figure 2.3. Activation loop phosphorylation is required for spatial restriction and activity of aPKC. (A-C and A'-C') *Inscuteable-GAL4* was used at 30°C and wandering third instar larvae were dissected. Anti-HA stains for the overexpressed proteins, p-410 labels the activation loop, p-560 labels the turn motif, and Miranda. (A-A') Wild type neuroblast during mitosis showing apical staining for both activation loop and turn motif specific antibodies, with basally restricted Miranda. (B-B') aPKC FL localizes apically showing proper activation loop and turn motif labeling, with basally restricted Miranda (C-C') aPKC T422A localizes around the entire cortex with reduced activation loop labeling, but maintenance of turn motif labeling and basally restricted Miranda. (D-E) Quantification of apical/cytoplasmic and apical/basal ratios for the overexpressed proteins using HA average intensities. *** Represents a P-value < 0.0001. ns represents no significance. Mean and SEM are plotted. (F) Apical/cytoplasmic ratios of p-410 labeling compared in aPKC FL and aPKC T422A overexpression. *** Represents a P-value < 0.0001. Mean and SEM are plotted. (G-I) Positively marked GFP MARCM clones of $FRT^{G13}aPKC^{K06403}$. (G) $FRT^{G13}aPKC^{K06403}$ clone with mislocalized Miranda and no aPKC. (H) aPKC FL is able to rescue Miranda basal localization in $FRT^{G13}aPKC^{K06403}$ null clones. (I) aPKC T422A is unable to rescue aPKC function in $FRT^{G13}aPKC^{K06403}$ null clones and still localizes around the entire cortex.

cortex of mitotic neuroblasts, colocalizing with Miranda at the basal cortex (Figure 2.4A). A small portion of metaphase neuroblasts showed reduced basal Miranda signal, consistent with aPKC T574A's higher *in vitro* activity. The pT422 specific antibody yielded a signal similar to the uniform cortical localization of aPKC T574A indicating that the protein contains the activation segment modification (Figure 2.4A). These neuroblasts exhibited significantly lower staining of pT574, however, consistent with the inability of the transgenically expressed protein to be phosphorylated at this site, but also indicating an effect on the endogenous protein (Figure 2.4A'). The decrease in the apical pT574 likely results from competition of aPKC T574A with endogenous aPKC for binding to the apical cortex. Although T574A is more catalytically active than T422A, it is still unable to rescue positively marked $aPKC^{k06403}$ clones, which may be due to its inability to be polarized (Figure 2.4F). These data demonstrate the necessity for turn motif phosphorylation in regulating aPKC's activity and localization.

Our results indicate that both the activation segment and turn motif must be phosphorylated for aPKC function during asymmetric cell division. Thus, the residual catalytic activity that proteins unphosphorylated at either of these sites possess is insufficient to polarize fate determinants, or the phosphorylations are necessary for activation of the autoinhibited conformation. Beyond their role in regulating aPKC activity, we have discovered that aPKC kinase domain phosphorylations are also important for aPKC polarity – if modification at either site is missing, aPKC is not restricted to the apical cortex. We also examined the localization of a commonly used "kinase dead" aPKC variant with a mutation in the ATP-binding pocket, K293W. This protein localized in a manner indistinguishable from the T422A and T574A proteins and

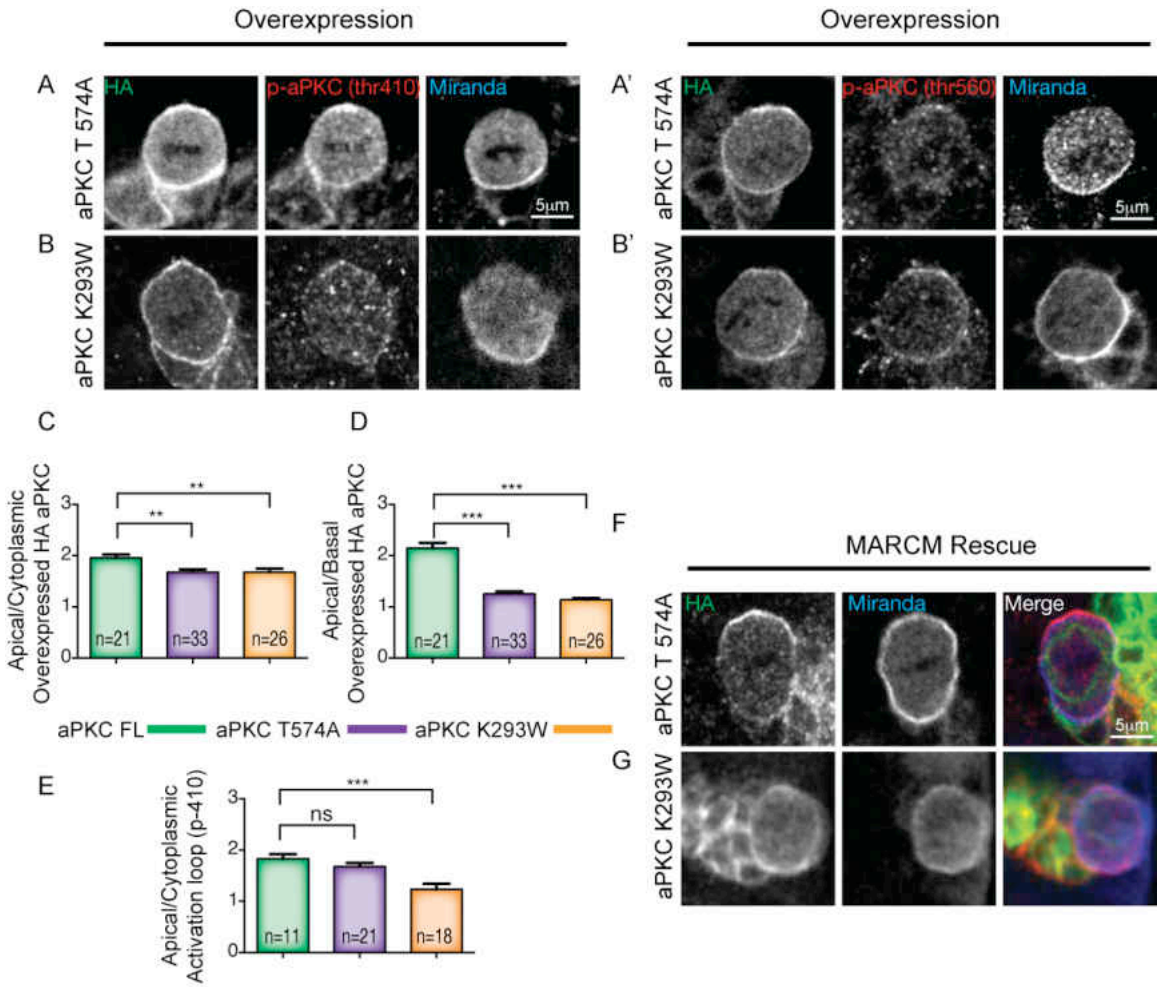


Figure 2.4. aPKC turn motif and ATP binding mutants are inactive and mislocalize. (A-B and A'-B') *Inscuteable-GAL4* was used at 30°C and wandering third instar larvae were dissected. Anti-HA stains for the overexpressed protein, p-410 for the activation loop, p-560 for the turn motif, and Miranda. (A-A') aPKC T574A showing complete cortical localization of the mutant protein with maintenance of activation loop labeling, reduced turn motif labeling, and basally restricted Miranda (B-B') aPKC K293W showing complete cortical localization of the mutant protein with reduced activation loop labeling, maintained turn motif labeling, and abnormal Miranda labeling. (C and D) Quantification of apical/cytoplasmic and apical/basal ratios for the overexpressed proteins using HA average intensities. *** Represents a P-value < 0.0001. ** Represents a P-value < 0.01. ns represents no significance. Mean and SEM are plotted. (E) Apical/cytoplasmic ratios of p-410 labeling compared in aPKC FL, aPKC T574A, and aPKC K293W overexpression. *** Represents a P-value < 0.0001. ns represents no significance. Mean and SEM are plotted. (F-G) Positively marked GFP MARCM clones of $FRT^{G13}aPKC^{K06403}$. (H) aPKC T574A is unable to rescue $FRT^{G13}aPKC^{K06403}$ null clone mislocalization of Miranda, while still weakly localizing around the entire cortex. (I) aPKC K293W is unable to rescue $FRT^{G13}aPKC^{K06403}$ null clone mislocalization of Miranda, while still localizing around the entire cortex.

was also unable to rescue *aPKC*^{k06403} mutant neuroblasts (Figure 2.4B-B' and G). In previous work, we found that the K293W mutation prevents phosphorylation of the T422 site (and likely also the T574 site), which has been observed with other PKCs (Graybill et al., 2012). Together, these results indicate that the kinase domain modifications regulate aPKC localization either through their role in promoting catalytic activity, or through some other unknown function.

Identification of a Par-independent aPKC cortical targeting mechanism

Polarization of aPKC occurs through coordinated interactions with the other Par complex members Baz and Par-6, and the Rho GTPase Cdc42. The interaction between aPKC and Par-6 is via heterodimerization of their PB1 domains (Atwood et al., 2007; Hirano et al., 2004; Noda et al., 2003; Rolls et al., 2003; Wodarz et al., 2000), while Baz interacts with the aPKC kinase domain as it is a substrate (Atwood et al., 2007; Joberty et al., 2000; Lin et al., 2000). A fundamental question in cell polarity is how these interactions are coordinated to properly polarize aPKC, the key output of the Par complex. To assess the role of these additional interactions in the ectopic localization of aPKC variants that lack phosphorylation at the activation segment and/or turn motif, we examined the localization of Baz and Par-6 in neuroblasts expressing T422A or T574A aPKCs. Surprisingly, Baz and Par-6 localization were unaffected by either of these proteins (Figure 2.5), indicating that the ectopic localization of aPKCs lacking kinase domain modifications occurs without the other components of the Par complex. Thus, we have identified a mechanism that targets aPKC to the cortex that is independent of the protein-protein interactions typically thought to be required for cortical recruitment.

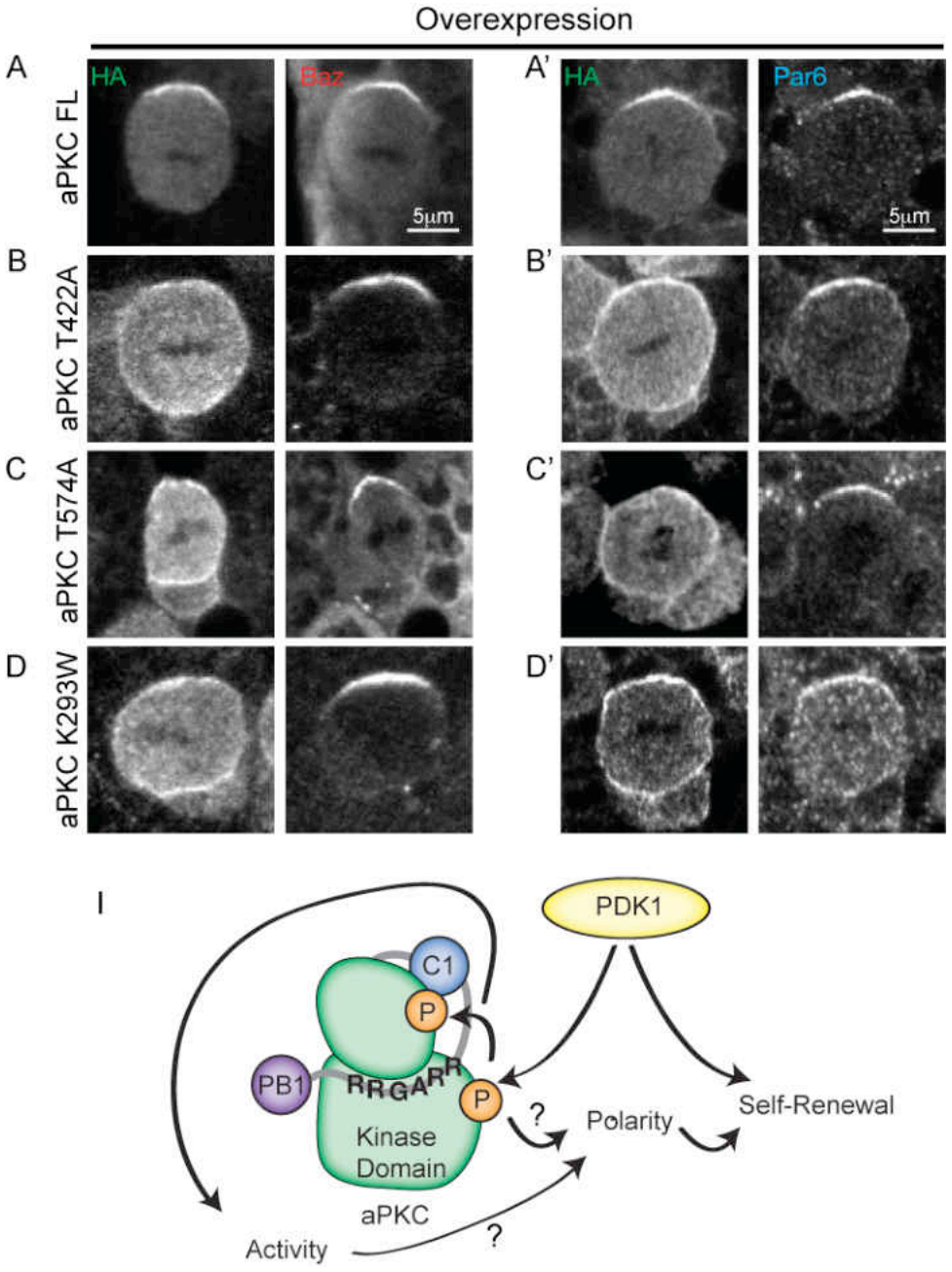


Figure 2.5. Kinase mutants localize around the cortex independent of other Par complex members. (A-E and A-E') *Inscuteable-Gal4* was used at 30°C and wandering third instar larvae were dissected. Anti-HA stains for the overexpressed protein. Neuroblasts were marked with Baz and Par-6. (A-E) Baz remains apically restricted regardless of mutant protein localization. (A'-E') Par-6 remains apically restricted regardless of mutant protein localization. (F) Model showing the possible roles of the activation loop phosphorylation within aPKC by PDK1, in generation of polarity and self-renewal.

DISCUSSION

We have found that PDK1 is necessary for proper development of the *Drosophila* central nervous system. Loss of PDK1 led to a reduction in neuroblast number, consistent with a defect in self-renewal. PDK1 has a known role as an upstream regulator of PKC family proteins, and we found that in neuroblasts lacking PDK1, aPKC had reduced activation segment phosphorylation. Surprisingly, however, aPKC was also mislocalized in the absence of PDK1 activity, targeting to the apical region, but also to the basal region where it colocalized with Miranda.

PDK1 is the upstream activating kinase for many AGC protein family kinases including Protein Kinase Cs (PKCs) (Newton, 2010). PKC phosphorylation by PDK1 can be important for catalytic competence, although the role of this modification in aPKC activity has been unclear (Newton, 2010; Pearce et al., 2010). Although a single substrate concentration *in vitro* enzyme assay suggested that activation segment phosphorylation by PDK1 is necessary for aPKC activity, a co-complex structure of the aPKC catalytic domain and a peptide from Par-3 lacked the modification, indicating that it is not absolutely essential for substrate binding (Wang et al., 2012). Our comprehensive analysis of aPKC variants lacking both activation segment and turn motif phosphorylations demonstrates that full activity requires modification at these sites, but significant residual activity remains in their absence.

Although the kinase domain phosphorylations are not absolutely required for catalytic activity, we have found that they are essential for aPKC function during neuroblast asymmetric cell division. Surprisingly, however, the modifications are not only essential for activity, as we see colocalization of the non-phosphorylatable aPKC

variants with Miranda, but for aPKC polarity. Apparently the phosphorylations normally prevent aPKC's entry into the basal domain, either via their role in enhancing catalytic activity, or through another function. A kinase-dead version of aPKC is also unpolarized, although the mutation that prevents catalytic activity does so by disrupting ATP binding, which has been found to be required for phosphorylation of both the activation segment and turn motif (Cameron et al., 2009). Analysis of neuroblasts expressing aPKC variants that selectively abrogate activity or kinase domain modification will be required to uncover the precise role of these modifications in aPKC polarization.

We found that much of the *pdk1* phenotype was recapitulated by the unphosphorylatable aPKC variants, although it is possible that PDK1 is important for self-renewal via other pathways. This possibility is supported by our observation that self-renewal was defective in *pdk1* RNAi neuroblasts even under conditions where sufficient aPKC activity remained for polarity. As PDK1 functions upstream of many other AGC protein kinases, one or more of these pathways could regulate self-renewal, potentially through a polarity-independent mechanism (Mora et al., 2004). The signaling pathways involved in maintaining stem-ness remain poorly understood and this system could be useful for uncovering this important aspect of stem cell function.

BRIDGE TO CHAPTER III

In the previous chapter, I described a role for aPKC activation loop and turn motif phosphorylations in the regulation of activity and localization of aPKC during asymmetric cell division of *Drosophila* neuroblasts. During this investigation it became clear that aPKC is able to localize to the cortex independently of other Par complex

members. This suggested that aPKC is able to interact with the cortex without binding to Par-3 and Par-6, at least in some mutant contexts. This interaction could be through yet to be determined protein-protein interactions, or through protein-lipid interactions, not yet appreciated for aPKC. In the following chapter, I describe how multiple domains within aPKC are working together in order to properly localize and activate aPKC. This mechanism involves the coordination between multiple protein-protein interactions, as well as a newly identified protein-lipid interaction. In the dividing *Drosophila* neuroblast, neither interaction is sufficient to localize or activate aPKC properly, indicating that protein-protein and protein-lipid interactions are required to properly generate polarity of aPKC.

CHAPTER III

LIPID AND PROTEIN INTERACTIONS COORDINATE LOCALIZATION AND ACTIVITY OF ATYPICAL PROTEIN KINASE C (APKC) DURING ASYMMETRIC CELL DIVISION

This chapter contains unpublished co-authored material

Author contributions: Michael Drummond, Scott X. Atwood, and Kenneth Prehoda designed the research; Michael Drummond and Scott X. Atwood performed the research; Michael Drummond, Scott X. Atwood and Kenneth Prehoda analyzed the data; and Michael Drummond, Scott X. Atwood and Kenneth Prehoda wrote the manuscript.

INTRODUCTION

Generation of molecularly distinct regions of a cell, cell polarity, is required for diverse processes such as cell migration, maintenance of tissue homeostasis, and regulation of cell fate decisions. Stem cells generate cell polarity to divide asymmetrically, separating proteins specifying proliferation from those involved in differentiation. This ensures that the stem cell will self-renew, a hallmark of all stem cells, and also give rise to a daughter cell that is able to differentiate into all other necessary cell types. Proper maintenance of this asymmetric division is essential for early development, as well as avoiding overproliferation that can lead to tumor-like phenotypes (Doe, 2008; Homem and Knoblich, 2012; Prehoda, 2009)

The Par complex consisting of Bazooka (Baz; Par-3), Cdc42, Par-6, and the serine/threonine kinase, atypical kinase C (aPKC), is essential for the establishment and

maintenance of the above-mentioned diverse polarities. In the *C. elegans* zygote, where these proteins were first discovered, this complex is localized to the anterior side of the cell and differentially partitions developmental factors during the first cell division (Kemphues et al., 1988; Suzuki and Ohno, 2006). *Drosophila* neural stem cells, referred to as neuroblasts (NBs), dynamically regulate the polarity of the Par complex during mitosis to ensure self-renewal and segregation of fate determinants to daughter cells. This is in contrast to epithelial cells, which maintain Par complex polarization throughout the cell cycle to preserve cellular architectures. Loss of Par complex localization in any of the aforementioned systems is detrimental to proper development and cellular homeostasis (Homem and Knoblich, 2012; Suzuki and Ohno, 2006; Tepass, 2012).

The Par complex mediates establishment of polarity by cortical exclusion, specifically through the kinase activity of aPKC (Atwood and Prehoda, 2009; Betschinger et al., 2003; Haenfler et al., 2012; Smith et al., 2007). In NBs, aPKC and Miranda (Mira) are localized to opposing sides of the cell. Mira is a scaffold protein for the fate determinant proteins, Brain tumor (Brat) and Prospero (Pros) that direct cellular differentiation (Doe et al., 1991; Ikeshima-Kataoka et al., 1997; Lee et al., 2006c). Upon division, the Par complex is localized to the apical side and prevents apical localization of Mira via phosphorylation (Atwood and Prehoda, 2009). This results in a self-renewed mother cell and a ganglion mother cell (GMC) that differentiates to populate the central nervous system with neurons and glia (Doe, 2008; Sousa-Nunes and Somers, 2013). This mechanism of polarization is not specific to Mira. The neoplastic tumor suppressor lethal giant larvae (Lgl) and a Notch regulator, Numb, are spatially regulated by aPKC in a similar manner (Betschinger et al., 2003; Smith et al., 2007).

Spatial regulation of aPKC is crucial for proper organism development, prevention of tissue disorganization, and overproliferation, yet the mechanisms that regulate its activity and localization remain unclear. Mutations in genes required for proper aPKC polarization are detrimental to organism survival. An intensely studied example is Lgl. In the developing central nervous system (CNS) of *Drosophila, lgl* loss of function mutants do not faithfully restrict aPKC to the apical cortex, resulting in extra NBs (Lee et al., 2006b). The same phenotype, albeit more dramatic, is observed when a constitutively active cortical aPKC (aPKC-CAAX) is overexpressed, causing fate determinants carried by Mira, and Numb to localize aberrantly, ultimately resulting in overproliferation of NBs (Haenfler et al., 2012; Lee et al., 2006b). These results demonstrate the necessity of spatially regulating aPKC, but mechanistic details of aPKC polarization are unclear.

Genetic and biochemical data have demonstrated that Baz is likely the first member of the complex to be localized to the apical cortex of embryonic NBs (Kuchinke et al., 1998). aPKC and Par-6 act downstream of Baz, since *aPKC* and *Par-6* mutants do not disrupt Baz apical restriction in NBs (Kuchinke et al., 1998; Petronczki and Knoblich, 2001; Rolls et al., 2003). Next, Cdc42 is either recruited or activated at the same location (Atwood et al., 2007). Par-6 interacts with Cdc42 allowing for aPKC polarization though heterodimerization of aPKC's and Par-6's PB1 domains (Atwood et al., 2007; Garrard et al., 2003; Noda et al., 2003). This would suggest that aPKC is polarized through its interaction with the Par-6 PB1 domain, but no functional role in polarization for other domains within aPKC is reported.

Here we examine the mechanism that regulates aPKC localization by determining the domains within aPKC required for localization and kinase activity, using *Drosophila* NBs as a model. We were able to determine that a simple model suggesting that the PB1-PB1 interaction between aPKC and Par-6 is insufficient to explain aPKC polarization. Instead, aPKC requires coordinated interactions between Cdc42, Par-6, and direct cortical binding of the C1 domain. We found that disrupting any of these interactions individually led to mislocalization of aPKC, and in some cases, Par-6 and Mira, during asymmetric cell division of NBs. This led us to propose a model where the localization mechanism of aPKC is coupled to its activation, and uncoupling them leads to fate determinant segregation defects.

MATERIALS AND METHODS

Fly strains and genetics

W^{1118} (wild type), *inscutable-GAL4* (Doe Lab), *UAS-HA:Par-6 FL, UAS-HA:Par-6 ISAA* (Atwood et al., 2007), *UAS-HA:aPKC ΔPB1* (107-606), *UAS-HA:aPKC PB1* (1-111), *UAS-HA:aPKC ΔPB1PS* (139-606), *UAS-HA:aPKC ΔC1* (lacking 141-196), *UAS-HA:aPKC RD* (1-195), *UAS-HA:aPKC C1* (139-195), *UAS-HA:aPKC FL* (1-606), *UAS-HA:aPKC D63A, UAS-HA:aPKC ΔPDZL* (1-600), (this study). Stocks were balanced over *TM3, Sb.* To produce *UAS-HA:aPKC* transgenic animals, we PCR amplified and subcloned the coding sequence into pUAST-attp vectors downstream of a 5' hemagglutinin (HA) tag and generated transformants using standard injection methods (Genetic Services). Wild type and UAS expressed larvae (*inscuteable-GAL4*) were aged at 30˚C until the wandering third instar larval stage.

MARCM clones were generated as previously described (Lee and Luo, 1999). In brief, *FRT^{G13}aPKC^{k06403}/Cyo* were crossed to *;;UAS-aPKC FL*, *;;UAS-HA:aPKC PB1*, and *;;UAS-HA:aPKC ΔC1* flies. F1s males lacking Cyo were collected and crossed to virgin *elav-GAL4, UAS-mCD8:GFP, hs:flp ; FRT^{G13}, tubPGal80* (Doe Lab). Larvae were heat shocked 24 hours after larval hatching at 37°C for 90 minutes and again 10-16 hours after the first heat shock. Larvae were allowed to grow to the wandering third instar larval stage at 22°C or 25°C and then dissected and immunostained. aPKC null clones were positively marked with GFP and the presence of the mutant aPKC was visualized by staining for HA.

Antibodies and immunostaining

We fixed and stained larval brains as previously described (Atwood and Prehoda, 2009). Primary antibodies used in this study included: rabbit anti-PKCζ (C20; 1:1000; Santa Cruz Biotechnology Inc), rat anti-Par-6 (1:1000) (Rolls et al., 2003), guinea pig anti-Mira (1:500) (Gift from the Doe Lab) rat anti-Mira (1:500) (Gift from the Doe Lab), rabbit anti-Phospho-Histone H3 (1:10,000; Santa Cruz), guinea pig anti-Baz (1:1000) (Gift from the Doe Lab), mouse anti-HA (1:500; Covance), rat anti-Deadpan (Gift from the Doe Lab), Chicken anti-GFP (1:5000; ab13970, Abcam). Secondary antibodies were from Jackson ImmunoResearch Laboratories and Invitrogen. Confocal images were collected using a Zeiss 700 equipped with 40x oil immersion objective/1.43 NA or an Olympus Fluorview 1000 equipped with a 40x oil immersion objective/1.30 NA. Figures were generated using Fiji, Adobe Photoshop, and Adobe Illustrator. Quantifications were

done using Graphpad Prism. For comparisons of apical/cytoplasmic ratios, we used a two-tailed unpaired t-test.

Protein purification and binding experiments

All GST and His tagged proteins were expressed and purified, as previously described (Atwood et al., 2007). All MBP-tagged proteins were purified, as described (Graybill et al., 2012). *Drosophila* embryonic lysate were prepared, as previously described (Atwood et al., 2007). Yeast two-hybrid assays were performed by cloning constructs into pGBK and pGAD plasmids and transfecting into yeast strain AH109. Yeast were plated onto SD(-LEU)(-TRP) and SD(-HIS)(-LEU)(-TRP) plates, incubated at 30°C for 96 hours, and assayed for growth.

Giant unilamellar vesicle production

All lipids were purchased from Avanti Polar Lipids. Lipids used in this study: L-a-phosphatidylcholine (840051C), L-a-phosphatidylserine (840032), L-a-phosphatidylinositol-4-phosphate (840045X), L-a-phosphatidylglycerol (840101C), and L-a-phosphatidic acid. Giant unilamellar vesicles (GUVs) were prepared as previously described (Moscho et al., 1996). In brief, lipids were added to 500 ml of chloroform in a round bottom flash and spun at 150 rpms in a 50°C for 2 minutes. Chloroform was removed by rotovap and placed in a vacuum chamber for 15 minutes. Lipids were then resuspended in 0.2 M sucrose at 0.5 mg/ml and incubated in a 50°C water bath for 4-16 hours. After incubation, GUVs were placed at 4°C for storage for up to 5 days.

Lipid binding cosedimentation assay

Cosedimentation was carried out by methods previously described (Prehoda et al., 2000). In brief, proteins were clarified by a pre-spin at 100,000 x g for 30 minutes using a TLA-100 at 10 C. Next, a final concentration of 5 μM protein was incubated with GUVs containing 0.23 mg/ml lipids in assay buffer (20 mM HEPES, pH 7.0, 50 mM NaCl, 1 mM DTT) and allowed to incubate for 10 minutes at room temperature. The reaction was then spun at 100,000 x g for 60 minutes using a TLA-100 rotor at 10°C. Supernatants were completely removed and saved for SDS-PAGE analysis. Pellets were resuspended in assay buffer and saved for SDS-PAGE analysis. All samples were run on 12.5% SDS PAGE gels and subsequently stained with coomassie. Gels were imaged with a scanner.

RESULTS

The aPKC PB1 domain is required but not sufficient for aPKC polarization

According to the current model, aPKC's PB1 domain is sufficient for polarization through heterodimerization with Par-6's PB1 domain and subsequent recruitment to the apical cortex through the small GTPase Cdc42 (Atwood et al., 2007; Noda et al., 2003; Prehoda, 2009). This model lacked functional details of the role other aPKC domains play in polarization, and in general lacked mechanistic details of how aPKC polarization occurs. Given the importance of aPKC in the generation and maintenance of polarity, we sought to understand the mechanistic details by which this occurs.

In order to dissect the underlying mechanism responsible for aPKC polarization, we performed an *in vivo* structure function study using *Drosophila* NBs. We were specifically interested in mitotic NBs, since this is the point in the cell cycle when polarity is generated. To accomplish this, we generated transgenic *Drosophila* that

contained the full-length protein (aPKC FL), the PB1 domain alone (aPKC PB1), or the deletion of the PB1 domain (aPKC ΔPB1) under the control of the *UAS-GAL4* system (Figure 3.1A). These were injected into the same chromosomal location (attp2) and each contained an NH_2-terminal hemagglutinin (HA) tag. Localization of all of the overexpressed transgenes was visualized by HA staining. In order to make conclusions about the role of individual domains within aPKC, we first determined if overexpressed full-length protein localized correctly. When we drove expression of aPKC FL in NBs using *inscuteable-GAL4*, we observed aPKC FL polarized to the apical cortex in a wild type manner without causing any dominant phenotypes throughout the cell cycle (Figure 3.1B, 3.1B', and data not shown). For quantification purposes, we measured the enrichment at the apical cortex, using a ratio of apical to cytoplasmic intensities of the HA antibody stains, during metaphase, for the overexpressed proteins (Figure 3.1F). This allowed us to unambiguously distinguish between apical enrichment and cytoplasmic localization. Next, we determined if our *aPKC FL* allele was able to rescue aPKC loss of function (*LOF*) phenotypes within *aPKC LOF* clones, where Mira is no longer restricted to the basal domain of mitotic NBs. This approach allows us to make conclusions about how our overexpressed proteins are functioning in the absence of endogenous aPKC. Our aPKC FL rescued the restriction of Mira to the basal cortex (Figure 3.1H). Since aPKC FL localized as expected and was able to rescue Mira localization in our *LOF* clone analysis, we determined that we would be able to test the current model of aPKC polarization using our transgenic flies.

To test the sufficiency of the PB1 domain, we overexpressed aPKC PB1 in NBs. We observed cytoplasmic localization of aPKC PB1 (Figure 3.1C), suggesting that

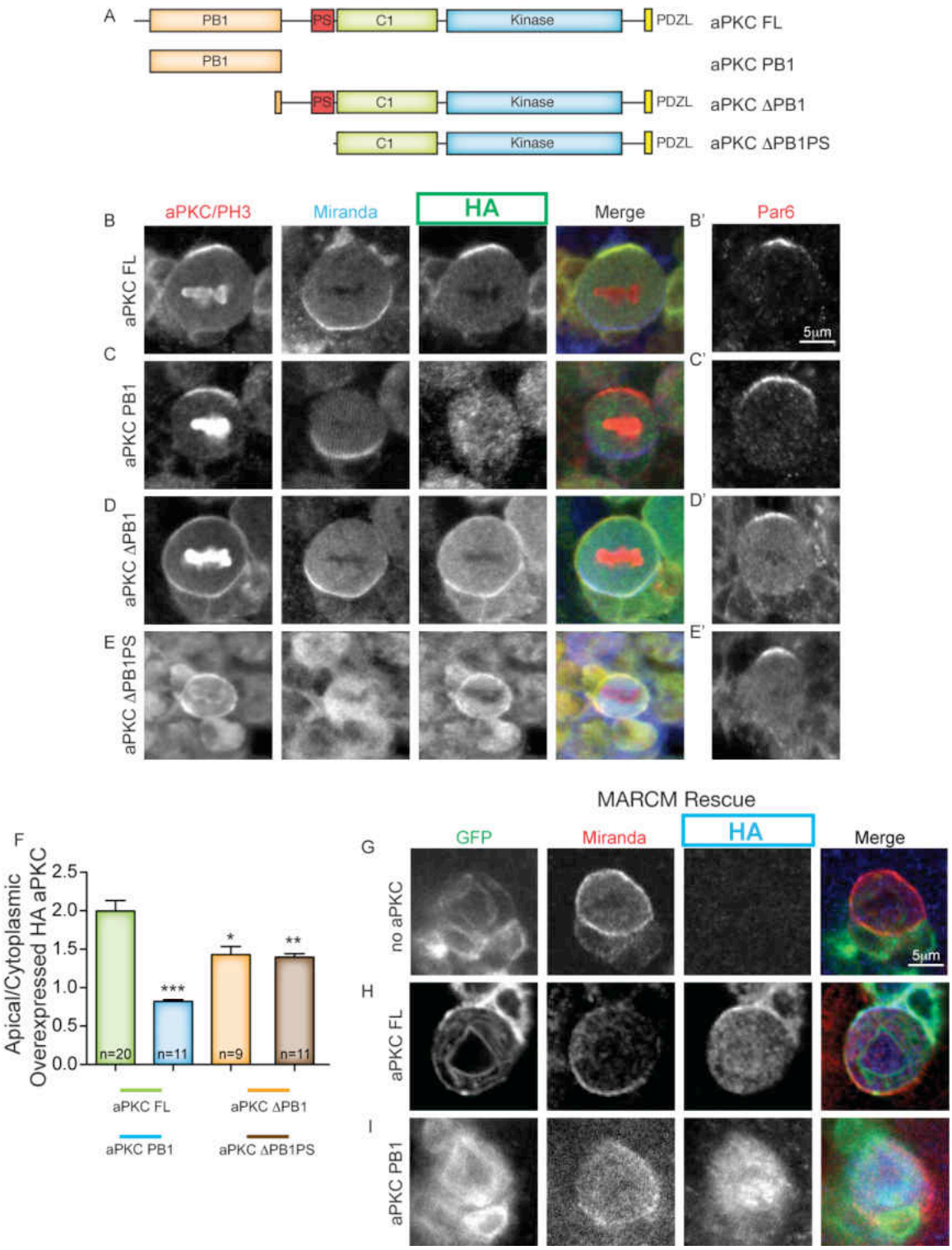
aPKC's PB1 domain is not sufficient for polarization. We did not observe any defects in endogenous Par-6 or Mira (Figure 3.1C and C'), suggesting that endogenous proteins were properly functioning, and our PB1 overexpression was not causing any noticeable dominant phenotypes. Since aPKC and Par-6 PB1 domains are sufficient to interact *in vitro* (Noda et al., 2003), it was possible that the endogenous aPKC bound to Par-6 with higher affinity, which was leading the overexpressed PB1 domain to localize in the cytoplasm. To verify that the PB1 domain was not sufficient for polarization, we assessed the localization of aPKC PB1 in *aPKC LOF* clones. Overexpressed aPKC PB1 localized to the cytoplasm in this genetic background, verifying that it is not sufficient for aPKC polarization (Figure 3.1I).

Next, we tested the requirement of the aPKC PB1 domain. We removed the PB1 domain from aPKC (aPKC ΔPB1) and investigated the localization as described above. Although the current model predicts that aPKC lacking its PB1 domain would localize to the cytoplasm due to it being the only know interaction it has with Par-6, it localized around the entire cortex in mitotic NBs (Figure 3.1D). This localization was independent of Par-6, as Par-6 maintains apical localization (Figure 3.1D'). These data suggest a general cortical signal exists within aPKC, outside of its PB1 domain. This localization signal is likely independent of aPKC's interactions with Par-6, since Par-6 remains apically localized. The observation that aPKC ΔPB1 domain localized around the entire cortex also implies that aPKC's PB1 domain could be suppressing an unidentified cortical targeting domain within aPKC. These data also reiterate that aPKC's interaction with Par-6 through their PB1 domains, while not sufficient, is necessary for apical polarization.

aPKC's PB1 domain is required for aPKC activation

Precise control of aPKC's kinase activity is required to ensure proper development, as well as prevention of tumorigenic overproliferation (Guyer and Macara, 2015; Lee et al., 2006b) aPKC's pseudosubstrate (PS) motif binds to the active site to prevent ectopic kinase activity, until needed (Newton, 2010). It has recently been demonstrated *in vitro,* that binding of Par-6 activates aPKC through displacement of its PS (Graybill et al., 2012). In organisms lacking aPKC or expressing an unphosphorylatable Mira, Mira localizes around the entire cortex (Atwood and Prehoda, 2009). This demonstrates that aPKC phosphorylation is required to restrict Mira to the basal cortex of mitotic NBs. We compared the localization of Mira in brains overexpressing aPKC ΔPB1 versus aPKC lacking the PB1 and PS (aPKC ΔPB1PS) in mitotic NBs to investigate the role of the aPKC PB1 domain and PS in activity.

Figure 3.1 (next page). aPKC's PB1 domain is required but not sufficient for polarization. (A) Cartoon depiction of aPKC domain architecture, showing a representation of the mutant transgenes overexpressed in this panel. (B-E) Mitotic larval neuroblast overexpressing the indicated transgenes using *inscuteable-GAL4* labeled with aPKC/PH3, Miranda and HA. HA represents the localization of the overexpressed proteins. Anti-aPKC also stains any overexpressed protein that contains the kinase domain. Scale bars represent 5 μm. (B'-E') Par-6 stains are from separate neuroblasts overexpressing the indicated mutant protein (B-B') aPKC FL showing apical localization with wild type Miranda and Par-6. (C-C') aPKC PB1 showing cytoplasmic localization of the mutant protein with wild type Miranda and Par-6. (D-D') aPKC ΔPB1 localizing around the entire cortex. Miranda and Par-6 maintain their wild type localization. (E-E') aPKC ΔPB1PS localizing around the entire cortex with displaced Miranda and wild type Par-6 labeling. (F) Apical/cytoplasmic intensity ratios for each of the overexpressed proteins labeled by HA, compared to overexpression of aPKC FL. The mean and SEM are plotted. *** p-value < 0.0001, ** p-value = 0.0031, and * p-value = 0.0135 (G-I) *aPKC LOF* clones positively marked with GFP, labeled with Miranda and HA, expressing the indicated transgene. (G) No aPKC expression with Miranda around the entire cortex. (H) aPKC FL overexpression rescues basal restriction of Miranda. (I) aPKC PB1 domain localizes in the cytoplasm of *aPKC LOF* clones.


A
PB1
PS
C1
Kinase
PDZL
aPKC FL
aPKC PB1
aPKC ΔPB1
aPKC ΔPB1PS
B
aPKC/PH3
Miranda
HA
Merge
B'
Par6
5μm
C
C'
D
D'
E
E'
F
Apical/Cytoplasmic Overexpressed HA aPKC
2.5
2.0
1.5
1.0
0.5
0.0
***
*
**
n=20
n=11
n=9
n=11
aPKC FL
aPKC ΔPB1
aPKC PB1
aPKC ΔPB1PS
MARCM Rescue
G
GFP
Miranda
HA
Merge
no aPKC
5μm
H
aPKC FL
I
aPKC PB1

Psuedosubstrates are known to play a role in repressing kinase activity of PKC family proteins (Newton, 2010). Therefore we predicted that removal of the PB1 through the PS had two likely outcomes. The PS could be required for both, cortical targeting and activity inhibition, leading to the overexpressed protein being active in the cytoplasm, or the PS is only required for kinase activity regulation, therefore it would localize around the entire cortex while being active. Ectopically expressed aPKC ΔPB1 co-localizes with Mira at the basal cortex (Figure 3.1D). The lack of Mira displacement by aPKC ΔPB1 demonstrates that the protein is inactive in the basal domain, even though it is bound to the cortex. Since aPKC ΔPB1 is cortical but inactive, this suggests that cortical binding may not be sufficient for aPKC activation in mitotic NBs. Consistently, the removal of the PB1 alone is not sufficient to activate aPKC *in vitro* (Graybill et al., 2012). Similar to aPKC ΔPB1, aPKC ΔPB1PS localized around the entire cortex, Par-6 remained apical, but Mira localized exclusively to the cytoplasm (Figure 3.1E and 3.1E'). This demonstrates that aPKC ΔPB1PS is active in the basal domain and likely along the entire cortex. This suggests that the PS is required to represses ectopic kinase activity, but it is not required for general cortical targeting. Further supporting this, we also observed a dramatic increase in NBs (100 vs >1000 per lobe, data not shown) consistent with aPKC ΔPB1PS being an active kinase. These data revealed that the PB1 domain of aPKC is required for both apical restriction of aPKC and activation *in vivo*, likely through Par-6 PB1 domain interaction.

The aPKC C1 domain is a cortical targeting signal

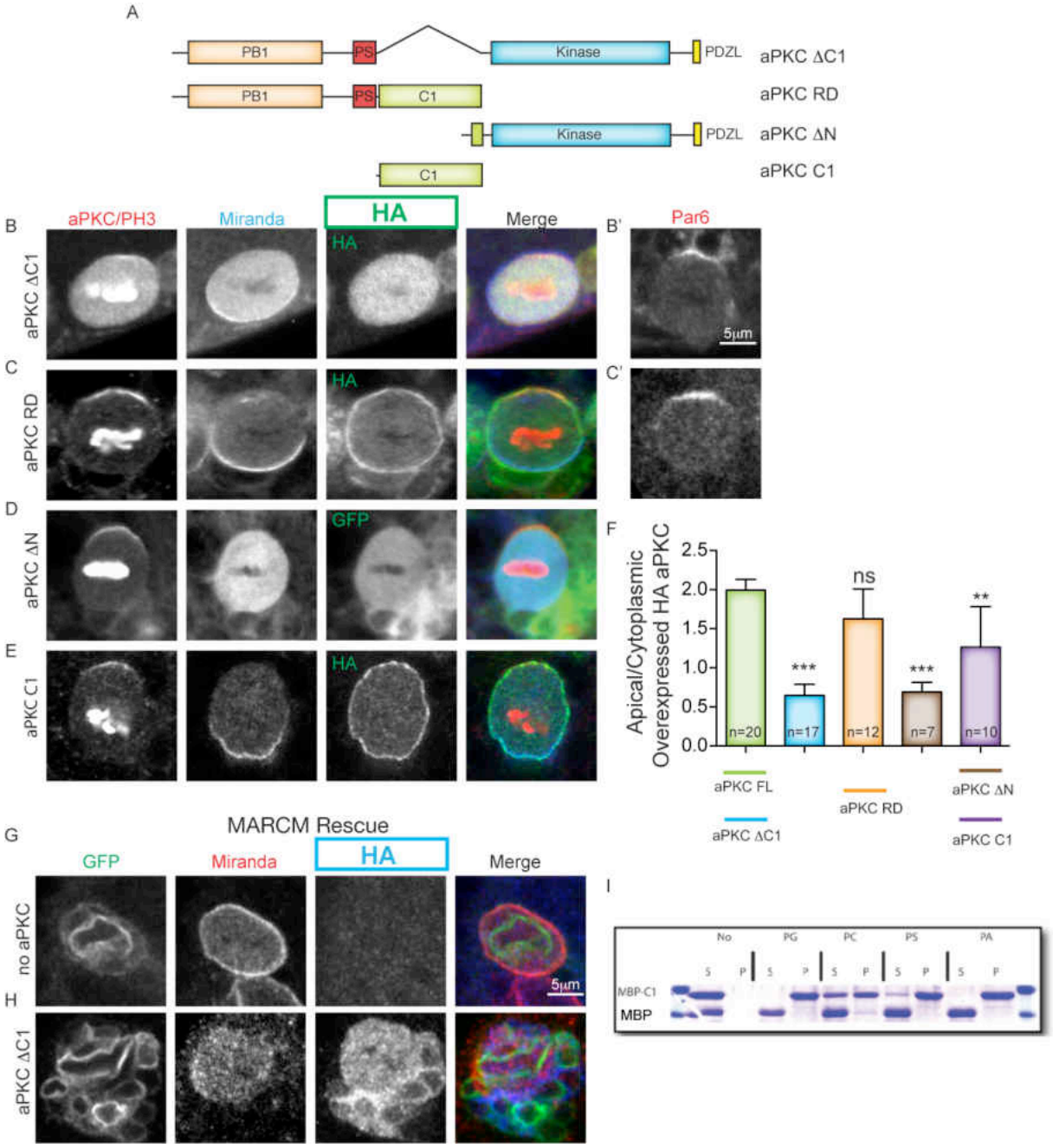
The cytoplasmic localization of aPKC PB1 and complete cortical localization of aPKC ΔPB1 indicated the presence of a cortical targeting signal within aPKC outside of the PB1 domain. In order to determine which domain(s) could be playing a role in cortical targeting, we removed candidate domains. We hypothesized that the C1 domain of aPKC was an excellent candidate. In canonical PKCs, C1 domains play an important role in membrane targeting and activation through binding of diacyglycerol (DAG) and phorbol esters (Newton, 2010). However, aPKC contains four arginine residues within its C1 domain, that render it insensitive to DAG (Pu et al., 2006). If the C1 domain of aPKC plays a role in localization, we expected that its removal would lead to cytoplasmic localization of aPKC.

When we overexpressed aPKC lacking its C1 domain (aPKC ΔC1) in NBs, cytoplasmic localization of the overexpressed protein was observed (Figure 3.2B). Endogenous Par-6 maintained wild type localization, while Mira showed enhanced cytoplasmic localization (Figure 3.2B and 3.2B'). These results are consistent with the previously described function of the C1 domain in playing a role in the regulation of aPKC kinase activity (Graybill et al., 2012; Lopez-Garcia et al., 2011). The same localization was observed for aPKC ΔC1 and Mira in *aPKC LOF* clones overexpressing aPKC ΔC1, verifying its importance (Figure 3.2H).

The cytoplasmic localization aPKC ΔC1 made the C1 domain a good candidate for cortical binding. To rigorously test this, we took advantage of a previously described aPKC allele in which the NH_2-terminal portion of the protein, through the C1 domain, is deleted (aPKC ΔN) (Lee et al., 2006b) and two new alleles, one that expresses the

regulatory domains in the NH_2-terminus of aPKC containing the PB1 through the C1 domain (aPKC RD) and the C1 domain alone (aPKC C1) (Figure 3.2A). When overexpressed, aPKC ΔN localized to the cytoplasm, while aPKC RD localized around the entire cortex of NBs (Figure 3.2C and 3.2D). Par-6 and Mira were undisturbed in aPKC RD expressing NBs (Figure 3.2C and 3.2C'), whereas Mira was displaced in aPKC ΔN expressing NBs, as previously reported (Lee et al., 2006b) (Figure 3.2D). Combining the data from these two constructs, we conclude that the C1 domain is likely responsible for general cortical targeting of aPKC. Out of the constructs tested thus far, all containing the C1 domain localized to the cortex, either apically or completely cortical, whereas any construct lacking the C1 domain localized to the cytoplasm. In order to verify the C1

Figure 3.2 (next page). **The C1 domain of aPKC is a general lipid binding domain required for aPKC cortical localization**. (A) Cartoon depiction of aPKC domain architecture, showing a representation of the mutant transgenes overexpressed in this panel. (B-E) Mitotic larval neuroblast overexpressing the indicated transgenes using *inscuteable-GAL4* labeled with aPKC/PH3, Miranda, and HA. Scale bars represent 5 μm. (B'-C') Par-6 stains are from separate neuroblasts overexpressing the indicated mutant transgene (B-B') aPKC ΔC1 localized to the cytoplasm with Miranda mostly displaced to the cytoplasm while Par-6 maintains wild type localization. (C-C') aPKC RD localized completely around the cortex with wild type Miranda and Par-6 localization. (D) aPKC ΔN localized to the cytoplasm and Miranda is displaced. (E) aPKC C1 localized to the entire cortex with wild type Miranda labeling. (F) Apical/cytoplasmic intensity ratios for each of the overexpressed proteins labeled by HA compared to overexpression of aPKC FL. ***p-value < 0.0001, **p-value = 0.0028, and ns is not significant. The mean and SEM are plotted. (G and H) *aPKC LOF* clones positively marked with GFP, labeled with Miranda, and HA while overexpressing the indicated transgene. (G) No aPKC expression with Miranda around the entire cortex. (H) aPKC ΔC1 localizes to the cytoplasm and Miranda is displaced into the cytoplasm. (I) Purified MBP:C1 domain binds to phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphatidylserine, and phosphatidic acid (PA) in a lipid cosedimentation assay while MBP does not. S represents the supernatant, the unbound fraction, while P represents the pellet, the bound fraction. No lipid indicates the absence of liposomes in the reaction mixture.


A
PB1
PS
Kinase
PDZL
aPKC ΔC1
PB1
PS
C1
aPKC RD
Kinase
PDZL
aPKC ΔN
C1
aPKC C1
B
aPKC/PH3
Miranda
HA
Merge
aPKC ΔC1
B'
Par6
5μm
C
aPKC RD
C'
D
aPKC ΔN
GFP
E
aPKC C1
F
Apical/Cytoplasmic Overexpressed HA aPKC
2.5
2.0
1.5
1.0
0.5
0.0
ns
**
***
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n=20
n=17
n=12
n=7
n=10
aPKC FL
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aPKC ΔN
aPKC ΔC1
aPKC C1
G
MARCM Rescue
GFP
Miranda
HA
Merge
no aPKC
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aPKC ΔC1
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No
PG
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MBP-C1
MBP

domain role in cortical targeting, we tested the localization of aPKC C1. Overexpressed aPKC C1 in NBs localized around the entire cortex, demonstrating that it is sufficient for cortical binding but not polarization (Figure 3.2E). We conclude that the C1 is a general cortical targeting domain.

The aPKC C1 domain binds directly, but non-specifically, to phospholipids

Next, we sought to determine what the C1 domain of aPKC binds to at the cortex. C1 domains are common among PKC family members, most binding to DAG and phorbyl esters to help localize and activate the proteins (Newton, 2010). We hypothesized that the C1 domain would bind to phospholipids. This was tested by using purified MBP:C1 domain in a liposome co-sedimentation assay. We generated giant unilamellar vesicles (GUVs) with 100% phosphatidyserine (PS), phosphatidycholine (PC), phosphotidylglycerol (PG), or phosphatidic acid (PA). When we tested the C1 domain for binding with phospholipids, we discovered that the domain bound to all lipids tested in the GUV assay, while MBP bound to none of the liposomes (Figure 3.2I). This suggests that C1 domain is a general lipid binding domain. This is consistent with the C1 localizing around the entire cortex of *Drosophila* NBs when overexpressed. From these data, we conclude that aPKC's C1 domain is able to non-specifically bind to the entire cortex through direct phospholipid interactions.

aPKC NH_2- and COOH-termini intramolecularly interact with one another

aPKC RD and aPKC ΔPB1PS revealed that both the NH_2 and COOH ends of aPKC are required to suppress the general cortical targeting by the C1 domain, as removal of either led to complete cortical localization of the mutant proteins. In order to restrict this localization, we hypothesized that aPKC is in a conformation in which the C1

cortical targeting ability is suppressed until interaction with regulatory proteins occurs. This suggested that aPKC could have intramolecular interactions that prevent ectopic localization and activation of aPKC until it interacts with Par-6. This is true for other PKC family members. The crystal structures of PKCiota, PKCbeta, and PKCepsilon revealed that the COOH-terminus is able to interact with various regions within each protein (Kheifets and Mochly-Rosen, 2007; Messerschmidt et al., 2005). In order to investigate this possibility, we performed yeast two-hybrid assay using the COOH-terminal end of aPKC (533-606) to search for intramolecular interactions. In this analysis, we found that any construct that contained the PB1 domain or PS interacted with the COOH-terminal aPKC fragment (3.3A). The interaction was the strongest with constructs that contained both the PB1 and PS. We verified these results with purified components. GST:aPKC 533-606 bound to GFP:aPKC 28-113 but not to GFP:aPKC 259-606 (Figure 3.3B). This interaction also occurs in the full-length protein as GST:aPKC 533-606 interacts with aPKC 1-600, in which the intramolecular interaction should be disrupted, but not with the full-length protein (Figure 3.3C). We conclude that aPKC intramolecular interactions keep it in an inactive state until it binds the regulator proteins, Par-6 and Cdc42.

aPKC contains a PDZ ligand that binds Par-6 in the presence of Cdc42

We next sought to determine how Par-6 and Cdc42 regulate the intramolecular interactions within aPKC. We predicted that Par-6 binding should disrupt aPKC's intramolecular interactions. This disruption should be required to generate the polarity

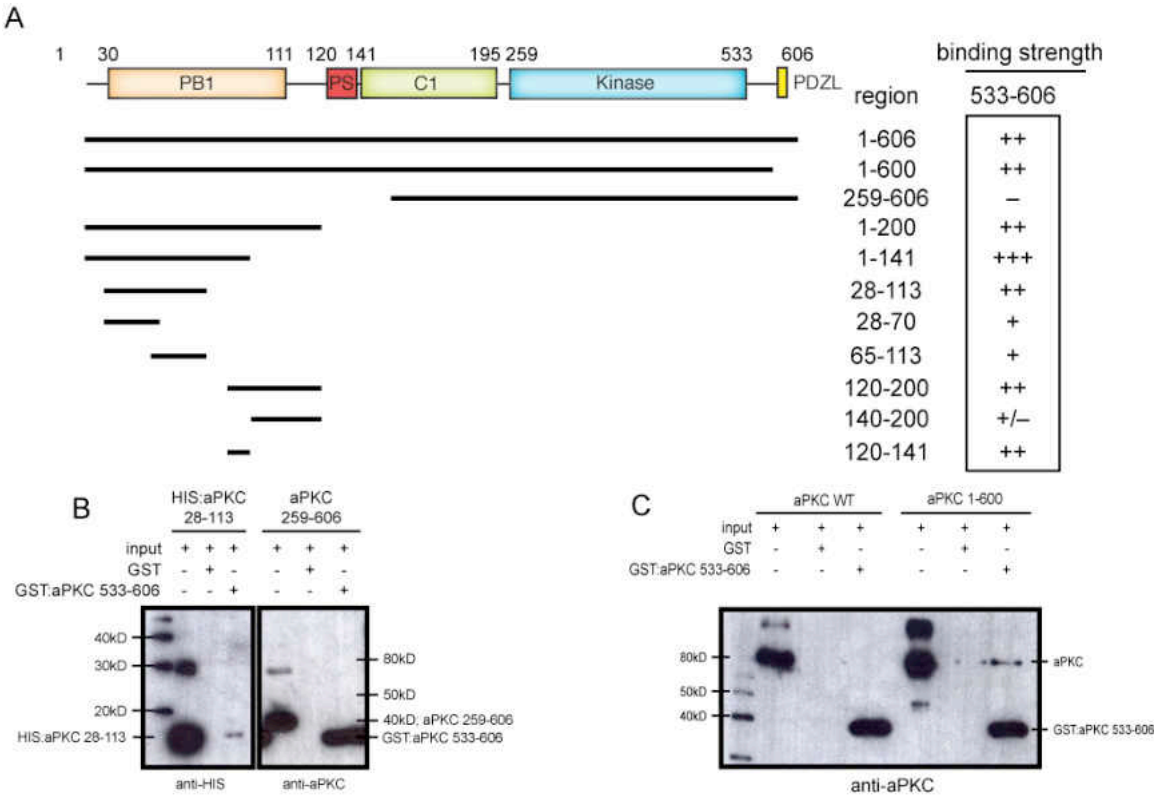

Figure 3.3. The NH_2- and COOH-termini of aPKC intramolecularly interact
(A) Yeast two-hybrid assay using aPKC 533-606 shows interaction with all constructs containing the PB1 domain (28-113) and the PS motif (120-141). A more robust interaction is observed when both the PB1 and PS domains are expressed together. (B) Western blot showing GST:aPKC 533-606 is able to bind aPKC PB1 but not the kinase domain (259-606) *in vitro*. (C) Western blot showing GST:aPKC 533-606 is able to bind aPKC with the intramolecular interaction disrupted (1-600) but not an aPKC where the intramolecular interaction is intact (wild-type 1-606).

and activate aPKC as previously observed *in vitro* (Graybill et al., 2012). To test this we bound GST: aPKC 533-606 to aPKC 1-600 and asked if Par-6 could disrupt this interaction. Indeed, Par-6 was able to disrupt the interaction, suggesting that it is able to disrupt the aPKC intramolecular interaction (Figure 3.4A). We next sought to determine if aPKC contained a conserved motif at its COOH-terminus that could aid in predicting what it may be binding. Upon alignment of aPKC's COOH-terminus with its human aPKC homologues and PKCalpha, we uncovered a conserved COOH-terminal valine,

which is a defining characteristic of COOH-terminal PDZ ligands (-D/E/K/R-X-ϕ-COOH; where ϕ = hydrophobic residue) (Figure 3.4B).

Since Par-6 contains a PDZ domain, we hypothesized it was a good candidate to bind aPKC's PDZ ligand. We tested this using pull-down experiments between GST:aPKC 533-606 that includes the PDZ ligand, and Par-6 lacking its PB1 domain (Par-6 CRIB-PDZ), since it is already known to dimerize with aPKC PB1 domain. GST:aPKC 533-606 was unable to pull-down Par-6 CRIB-PDZ (Figure 3.4C). We rationalized that interaction might require Cdc42, because it has been previously reported that Cdc42 can induce Par-6 binding to COOH-terminal PDZ ligands with higher affinity than on its own (Penkert et al., 2004; Peterson et al., 2004). Upon addition of Cdc42•GTPγS to our binding reaction, we observed Par-6 bound to the COOH-terminus of aPKC (Figure 3.4C). This suggests that Cdc42 promotes binding between Par-6 PDZ and the PDZ ligand of aPKC. This interaction likely occurs *in vivo* as well, as GST:Par-6 CRIB-PDZ was able to pull down aPKC from embryonic lysates supplemented with Cdc42•GTPγS (Figure 3.4D). This suggests a model in which aPKC, Par-6, and Cdc42 require coordinated interactions to form a complex. These interactions include, but may not be limited to, the PB1-PB1 interaction of Par-6 and aPKC, and a PDZ-PDZ ligand interaction between aPKC and Par-6, which requires GTP bound Cdc42.

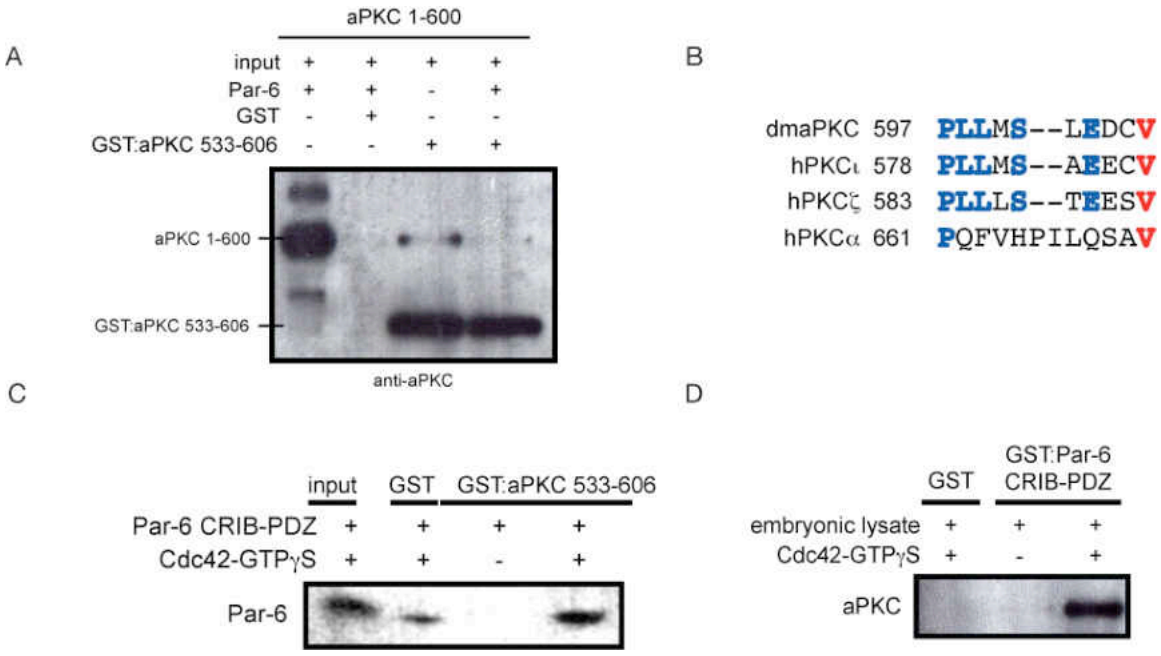

Figure 3.4. Par-6 regulates aPKC's intramolecular interaction and binds aPKC's PDZ ligand in a Cdc42 dependent manner. (A) Western blot demonstrating that Par-6 inhibits the interaction between GST:aPKC 533-606 and aPKC 1-600. (B) Alignment of *Drosophila* aPKC with human aPKCs and PKC alpha. (C) Western blot of GST:aPKC 533-606 interacting with Par-6 CRIB-PDZ in a Cdc42-dependent manner. (D) Western blot showing Par-6 CRIB-PDZ interacting with endogenous aPKC in a Cdc42-dependnet manner in embryonic lysates.

Interaction between aPKC PDZ ligand and Par-6 PDZ is required for proper polarity in neuroblasts

Next we sought to determine the importance of these interactions for generating polarity during NB divisions. In order to accomplish this, we generated an allele that blocked the PB1-PB1 dimerization of aPKC and Par-6, but did not activate the C1 domains cortical targeting observed when we completely removed the PB1 domain. To do this, we generated a transgenic line expressing an HA-tagged aPKC with a point mutation in aPKC's PB1 domain, D63A, that is known to inhibit Par-6 and aPKC PB1 dimerization (Hirano et al., 2004; Noda et al., 2003) (Figure 3.5A). When we overexpressed this protein in NBs, aPKC D63A localized to the cytoplasm without

disrupting Par-6 or Mira localization (Figure 3.5B and 3.5B'). This reiterates that PB1-PB1 interaction between aPKC and Par-6 is required to allow for aPKC localization to the apical cortex and could be required for PDZ-PDZ ligand interaction between aPKC and Par-6. This also suggests that the PB1-PB1 binding between aPKC and Par-6 is likely required to allow for C1 cortical targeting.

In order to test the requirement of PDZ-PDZ ligand interaction, we generated a transgenic line to express aPKC lacking its PDZ ligand (aPKC ΔPDZL). This overexpressed allele localized to the cytoplasm, indicating that the PDZ-PDZ ligand interaction is required for proper aPKC localization (Figure 3.5C). Overexpression of this allele also led to endogenous Par-6 localizing to the cytoplasm and Mira localizing around the entire cortex (Figure 3.5C and 3.5C'). This indicated that the PDZ-PDZ ligand interaction between aPKC and Par-6 is required to generate polarity, likely though an induced conformational change induced by Cdc42 binding. Since the PDZ-PDZ ligand interaction requires the presence of Cdc42 *in vitro*, we tested this in NBs as well. In order

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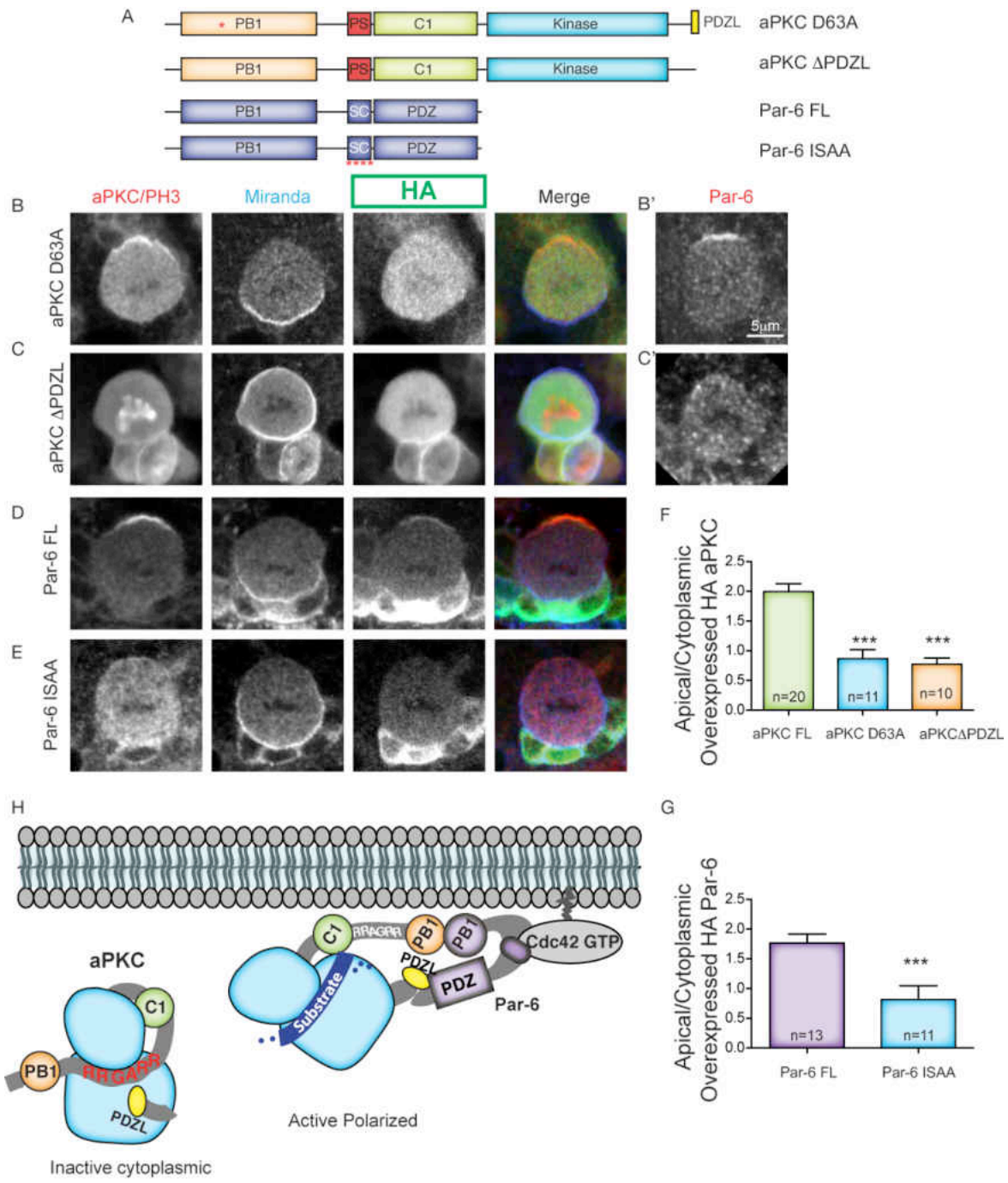
Figure 3.5 (next page). The PDZ ligand-PDZ interaction between aPKC and Par-6 governed by Cdc42 is required for neuroblast polarity. (A) Cartoon depiction of aPKC domain architecture, showing a representation of the mutant transgenes overexpressed in this panel. Red asterisks represent point mutations. (B-E) Mitotic larval neuroblast overexpressing the indicated transgenes using *inscuteable-GAL4* labeled with aPKC/PH3 or aPKC alone, Miranda, and HA. Scale bars represent 5 μm. (B-B') aPKC D63A localized to the cytoplasm and no defects were observed in Par-6 or Miranda localization. (C-C') aPKC ΔPDZL localized to the cytoplasm. Par-6 apical crescents are severely reduced and Miranda is uniformly cortical. (D) Par-6 FL localized apically and did not disrupt aPKC or Miranda staining. (E) Par6 ISAA localized to the cytoplasm. aPKC crescents were severely reduced and Miranda localization extended around the cortex. (F) Apical/cytoplasmic intensity ratios for each of the ectopically expressed proteins labeled by HA compared to overexpression of aPKC FL. *** p-value < 0.0001. The mean and SEM are plotted. (G) Apical/cytoplasmic intensity ratios for Par-6 FL versus Par6 ISAA HA signal. *** p-value < 0.0001. The mean and SEM are plotted. (H) Proposed model of aPKC polarization during asymmetric cell division of *Drosophila* neuroblasts.


A
PB1
PS
C1
Kinase
PDZL
aPKC D63A
aPKC ΔPDZL
PDZ
Par-6 FL
Par-6 ISAA
B
aPKC/PH3
Miranda
HA
Merge
B'
Par-6
aPKC D63A
5μm
C
C'
aPKC ΔPDZL
D
Par-6 FL
E
Par-6 ISAA
F
Apical/Cytoplasmic Overexpressed HA aPKC
n=20
n=11
n=10
***
aPKC FL
aPKC D63A
aPKCΔPDZL
G
Apical/Cytoplasmic Overexpressed HA Par-6
n=13
n=11
Par-6 FL
Par-6 ISAA
H
aPKC
C1
PB1
RRAGAPR
PDZL
Inactive cytoplasmic
Substrate
Cdc42 GTP
PDZ
Par-6
Active Polarized

to test this, we used previously described alleles, a full-length HA-tagged Par-6 (Par-6 FL) and a HA-tagged Par-6 ISAA, which has mutations in the 'Semi-CRIB' preventing its binding to Cdc42 (Atwood et al., 2007). Overexpressed Par-6 FL localized apically, as seen by HA staining, while aPKC remained robustly apical with polarized Mira (Figure 3.5D). Overexpressed Par-6 ISAA localized cytoplasmically in dividing NBs (Figure 3.5E). Overexpression of Par-6 ISAA also led to Mira ectopically localizing around the entire cortex and a concomitant reduction in aPKC apical staining (Figure 3.5E). This indicates that Par-6 must interact with Cdc42 in order to get aPKC to the cortex. We conclude that Par-6 and aPKC need to interact through both, PB1-PB1 heterodimerization, as well as, PDZ-PDZ ligand interaction, which require Cdc42 binding of Par-6. These interactions are necessary to restrict aPKC C1 domain cortical targeting to the apical cortex. Disruption of any of these interactions is sufficient to perturb polarity, and therefore, they must be tightly regulated.

DISCUSSION

aPKC is essential for the generation and maintenance of several cellular polarities, which are crucial to proper development and disease prevention. These diverse polarities require aPKC kinase activity to be restricted to specific areas of the cell. Moreover, loss of aPKC regulation can lead to premature loss of neural stem cells or disease states, such as cancer. Despite the overall importance of aPKC during development, the mechanism by which aPKC activity is spatially regulated remained poorly understood. Here, we demonstrate that the aPKC PB1-PB1 interaction Par-6, while required for apical localization in NBs, is not sufficient to explain how aPKC becomes polarized. Instead, aPKC polarization requires the precise regulation both inter- and intramolecular, protein-

protein interactions, as well as protein-lipid interactions. We were able to demonstrate that aPKC's NH_2- and COOH-termini interact. This intramolecular interaction is needed to suppress the complete cortical targeting activity of the aPKC C1 domain binding of phospholipids. In order to break aPKC's intramolecular interaction, Par-6 dimerizes with aPKC through PB1-PB1 interactions. This interaction allows for Par-6 to bind to aPKC's COOH-terminal PDZ ligand, which requires Cdc42. This set of interactions governs aPKC localization, and if any of them are disrupted, polarity within a dividing NB is compromised (Figure 3.5H).

This mechanism of aPKC polarization is likely to be similar in other model systems, as Cdc42, Par-6, and aPKC are required for polarity in diverse tissues. While similar, it will be interesting to investigate the differences. For example, in NBs, this complex acts downstream of Baz, while in some epithelial cells Cdc42 is upstream of Baz (Atwood et al., 2007; Tepass, 2012). Also, epithelial cells have multiple Par-6 PDZ ligand proteins, such as Crumbs and Stardust, which may be playing competing roles to regulate aPKC localization and kinase activity (Tepass, 2012). Furthermore, we did not exclude the possibility of aPKC's PDZ ligand interacting other PDZ domains, which are plentiful. Further investigation of other model systems will need to be done in order to elucidate the difference between cell types and organisms. These differences could help to explain why in some cancers aPKC is upregulated to drive tumorigenesis, whereas in others, aPKC loss leads to the upregulation of cell proliferation and tumor progression (Atwood et al., 2013; Guyer and Macara, 2015; Llado et al., 2015). It will be very important to identify these underlying differences in order to understand how to target aPKC activity as a therapy in these various disease states.

CHAPTER IV

SUMMARY AND CONCLUDING REMARKS

SUMMARY

The establishment and maintenance of cell polarity is required for a myriad of processes during development, as well as during adulthood of organisms (Homem and Knoblich, 2012; Suzuki and Ohno, 2006; Tepass, 2012). In epithelial cells these include barrier functions and nutrient absorption. Stem cells can use polarity to asymmetrically divide, generating a self-renewing cell, as well as a daughter cell that go on to assume a particular fate based upon the intrinsic and extrinsic signals it receives (Doe, 2008). Loss of cell polarity, in both types of cells, is implicated in the development and progression of a wide variety of cancers. *Drosophila* neural stem cells, neuroblasts, divide asymmetrically to self-renew and generate a ganglion mother cell that differentiates into neurons or glial to populate the developing central nervous system. They accomplish this by taking advantage of the evolutionarily conserved Par complex composed of Bazooka, Par-6, and aPKC, in close concert with the small Rho GTPase, Cdc42 (Sousa-Nunes and Somers, 2013). This complex is able to generate polarity in neuroblasts through the kinase activity of aPKC. aPKC segregates fate determinant and proliferation regulator proteins to opposite side of the cell as itself, through a phosphorylation induced cortical displacement mechanism (Prehoda, 2009). While much is known about how aPKC is able to polarize its substrates, the mechanisms that govern aPKC activity and restricted localization are yet to be fully elucidated.

PKC family members are regulated by post-translational modification. Of particular interest to this thesis is the phosphorylation of the activation loop by PDK1 and

subsequent autophosphorylation of the turn motif (Newton, 2010). The requirement for these phosphorylations in regulating aPKC was unclear, with some data suggesting they may not be required (Ranganathan et al., 2007; Wang et al., 2012). In Chapter II, I report that PDK1 is necessary for proper development of the *Drosophila* central nervous system. Knockdown, specifically in neuroblasts, led to a reduction of neuroblasts. Upon closer inspection of individual dividing neuroblasts, loss of aPKC activation loop phosphorylation was observed, suggesting the reduction in neuroblasts could, at least in part, be due to misregulation of aPKC activity. With the use of a highly quantitative *in vitro* kinase activity assay, we observed severely reduced kinase activity with the greatest difference being lower k_{cats}, when we mutated either the activation loop or the turn motif. In order to investigate if the *in vitro* observed reduced activity was physiologically relevant; we generated transgenic *Drosophila* that could express proteins with a non-phosphorylatable activation loop or a non-phosphorylatable turn motif in neuroblasts. We were able to determine that the activation loop and turn motif phosphorylations are critical for the regulation of localization and function of aPKC. Surprisingly, all the mutant proteins localized around the entire cortex in neuroblasts and did so independently of Baz and Par-6. This suggested that further studying the mechanisms involved in polarization of aPKC was needed.

In embryonic neuroblasts, Baz is the first protein to polarize (Kuchinke et al., 1998). Cdc42 acts downstream of Baz, and is required to recruit Par-6 and aPKC to the apical cortex (Atwood et al., 2007). In order to polarize, Par-6 and aPKC interact through their PB1 domains while Par-6 interacts with Cdc42 through Par-6 CRIB-PDZ (Garrard et al., 2003; Hutterer et al., 2004; Noda et al., 2003). Precise control of aPKC localization

and activity is required in neuroblasts to ensure proper development of the central nervous system within *Drosophila* larvae. Loss of aPKC in this model system leads to self-renewal defects, whereas inappropriate aPKC activity can lead to dramatic overproliferation and organismal death (Lee et al., 2006b; Rolls et al., 2003). In Chapter III, I uncovered various aPKC mutant proteins that were able to localize ectopically around the cortex independent of Par-6, again suggesting the existence of alternative modes of aPKC cortical binding. This cortical binding was previously unappreciated as no known information about domains outside of the PB1 domain of aPKC playing a role in polarization have been mechanistically investigated.

The previous model of aPKC polarization predicted that the aPKC PB1 domain would be sufficient for polarization through interaction with the Par-6 PB1 and Par-6's interaction with Cdc42. This was not the case, as expression of aPKC's PB1 domain alone was unable to polarize. The old model also predicted that removal of the PB1 domain from aPKC would lead to its cytoplasmic localization. To the contrary, removal of the PB1 domain led to completely cortical localization of the overexpressed mutant aPKC. While the mutant was completely cortical, it was not able to displace Mira from the basal cortex, indicating that it was inactive in the basal domain, likely because that PS was still bound to the kinase domain. This reiterated the point that aPKC contains a domain outside of its PB1 domain that is able to directly or indirectly get aPKC to the cortex. We identified this domain as the C1 domain, as it was sufficient to bind to phospholipids at the cortex, albeit in an unpolarized manner. It was determined that both the NH_2- and COOH-termini of aPKC are necessary to suppress ectopic localization of the C1 domain, suggesting the possibility of intramolecular interactions in aPKC. Indeed,

we identified that aPKC NH_2-terminal PB1/PS domains were able to interact with the COOH-terminus of the protein. Par-6 binding disrupts this interaction to at least partially activates aPKC (Graybill et al., 2012) and generate neuroblast polarity.

Alignment of the COOH-terminus of aPKC with human aPKC homologs demonstrated that aPKC contains a COOH-terminal PDZ ligand. This PDZ ligand interacts with the Par-6 PDZ domain, but only when GTP bound Cdc42 is present. This suggested a new model where aPKC interacts with Par-6 through PB1-PB1 heterodimerization and through PDZ ligand-PDZ interaction that is regulated by Cdc42. These interactions must be coordinated to restrict the general cortical targeting C1 domain to the apical cortex. This provides an elegant, albeit, slightly complex model for coupling aPKC localization and activation. Disruption of any of the newly identified protein-protein or protein-lipid interactions leads to improper polarity generation and subsequently improper segregation of cell fate determinants.

CONCLUDING REMARKS

My dissertation focused on the polarization of aPKC in neuroblasts, but it is likely that this core mechanism described here will apply to a wide range of organisms and tissues. This is likely since Cdc42 recruits Par-6 and aPKC in a variety of organismal models (Aceto et al., 2006; Atwood and Prehoda, 2009; Joberty et al., 2000; Lin et al., 2000). However, tissue specific modulators of this core mechanism are also likely to be present. For example, epithelial cells utilize many PDZ domain containing proteins to establish polarity (Tepass, 2012). Cross talk between these PDZ containing proteins with aPKC PDZ ligand, or competition for Par-6 PDZ domain, or both are likely. These

combinatorial interactions will possibly act to regulate aPKC localization and kinase activity, therefore be crucial to the generation of polarity. In fact, it has already been demonstrated that the Par-6 PDZ domain interacts with the apical epithelial proteins Stardust and Crumbs, and these interactions are necessary for the establishment and maintenance of polarity (Hurd et al., 2003; Kempkens et al., 2006; Lemmers et al., 2004). These interactions can lead to differential localization of aPKC-Par-6 and Baz in some tissues. For example, aPKC phosphorylation of Baz, in *Drosophila* follicular epithelial cells, in combination with Crumbs competing with Baz for Par-6 binding, leads to apical localization of aPKC-Par-6, and sub apical Baz (Morais-de-Sá et al., 2010). How these interactions might influence aPKC and Par-6 PDZ ligand-PDZ interaction is yet to be determined, but they are likely to play a role in both activation and localization. This is in contrast to *Drosophila* neuroblasts, where Baz, aPKC, and Par-6 all colocalize, highlighting the necessity to further investigate the mechanisms governing aPKC activity and localization in a variety of cell types.

Nonetheless, the newly identified mechanistic details identified through my dissertation work provide a core framework with which to move forward.

Intriguingly, aPKC has recently been identified to signal through important cell fate decision pathways such as Wnt, JAK/Stat, and Hedgehog (Atwood et al., 2013; Guyer and Macara, 2015; Llado et al., 2015). These signaling events are often referred to as polarity-independent, suggesting alternative modes of aPKC regulation. Our understanding of the molecular mechanisms that control aPKC in these newly identified processes is still in its infancy, but it is clear that the atypical members of the PKC kinase

family are going to be important therapeutic targets (Antal et al., 2015; Atwood et al., 2013; Justilien et al., 2014).

aPKC can act as both an proto-oncogene, and a tumor suppressor gene, suggesting it will vitally important to fully understand the mechanisms that govern its regulation before attempting to use aPKC activity modulators as therapeutics (Antal et al., 2015; Parker et al., 2014). For example, treatment of BCCs and LSCCs, in which over activation of aPKC can to lead to tumorigenic feed back loop through Hedgehog signaling, should be treated differently than when loss of aPKC activity leads to overproliferation of intestinal stem cells through increased Wnt signaling (Atwood et al., 2013; Justilien et al., 2014; Llado et al., 2015). Also, simply determining the level of aPKC expression in a tissue does not always correlate with the amount of aPKC activity. To the contrary, it was recently revealed that even though many disease states have abnormally high amounts of PKC expression, there is actually less activity, because the highly expressed protein is usually a mutant lacking activity (Antal et al., 2015).

As a first step to dissecting the mechanism of aPKC regulation in these pathways, it should be determined if canonical aPKC regulators like Cdc42, Par6, and Par-3 are also playing a role, in order understand if these are truly polarity-independent signaling events. Determining how aPKC is regulated in these new signaling pathways will allow for a more mechanistic approach to choosing therapeutics that either upregulate or downregulate aPKC activity, depending on the cellular context, and could lead to higher efficacy of these treatments.

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